PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12P 17/02, 1/00, C12N 5/02
C12N 5/04, C07D 305/14
A61K 31/335

A1

(11) International Publication Number: WO 93/23555

(43) International Publication Date: 25 November 1993 (25.11.93)

(21) International Application Number: PCT/US93/04424

(22) International Filing Date: 11 May 1993 (11.05.93)

(30) Priority data:
07/886,619 21 May 1992 (21.05.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/886,619 (CIP)
Filed on 21 May 1992 (21.05.92)

(71) Applicant *(for all designated States except US)*: THE PENN STATE RESEARCH FOUNDATION [US/US]; 114 Kern Graduate Building, University Park, PA 16802 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : ARTECA, Richard, N. [US/US]; 455 Westgate Drive, State College, PA 16803 (US). WICKREMESINHE, Enaksha [LK/US]; 6H Graduate Circle, State College, PA 16801 (US).

(74) Agent: MONAHAN, Thomas, J.; Intellectual Property Office, The Pennsylvania State University, 114 Barbara Building II, University Park, PA 16802 (US).

(81) Designated States: AU, CA, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: CULTURED *TAXUS* TISSUES AS A SOURCE OF TAXOL, RELATED TAXANES AND OTHER NOVEL ANTI-TUMOR/ANTI-VIRAL COMPOUNDS

(57) Abstract

Successful culture methods have been developed which result in stable, long-term tissue cultures derived from *Taxus* explants and hydroponically grown roots. These cultures offer a rapidly reproducible, continuously-available source for the production of purified taxol and taxol-related compounds. Culture methods include *in vitro* tissue culture and hydroponics. Cultures are initiated with stem or root tissues of *Taxus* or from roots grown hydroponically. Taxol production may be scaled to commercial levels by use of bioreactors. Screening assays are provided for species and cultures of *Taxus* that are sources of taxol and taxol-related compounds. In addition to obtaining the same compositions as presently directly extracted from yew trees, new compositions exhibiting taxol-like activity, have been purified from the novel *Taxus* sources, offering new horizons for chemotherapeutic agent development.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NL**	Netherlands
BE	Belgium	**GN**	Guinea	**NO**	Norway
BF	Burkina Faso	**GR**	Greece	**NZ**	New Zealand
BG	Bulgaria	**HU**	Hungary	**PL**	Poland
BJ	Benin	**IE**	Ireland	**PT**	Portugal
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SK**	Slovak Republic
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SN**	Senegal
CM	Cameroon	**LI**	Liechtenstein	**SU**	Soviet Union
CS	Czechoslovakia	**LK**	Sri Lanka	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**MC**	Monaco	**UA**	Ukraine
DK	Denmark	**MG**	Madagascar	**US**	United States of America
ES	Spain	**ML**	Mali	**VN**	Viet Nam
FI	Finland	**MN**	Mongolia		

"Cultured *Taxus* Tissues as a Source of Taxol, Related Taxanes and Other Novel Anti-tumor/Anti-viral Compounds"

This is a continuation-in-part of U.S. Serial No. 07/886,619, filed May 21, 1992, the contents of which are incorporated by reference.

BACKGROUND OF THE INVENTION

This invention relates to cultured *Taxus* tissues as a source of taxol, 10-deacetyl baccatin III, 7-epi-10-deacetyl baccatin III, baccatin III, 7-epi baccatin III, 9-dihydro-13-acetyl baccatin III, cephalomannine, 10-deacetyl taxol, 7-epi10-deacetyl taxol, 7-epi taxol, other known and novel natural derivatives of these compounds, and other related taxanes including precursors and intermediates in the biochemical pathway resulting in taxol production, and active derivatives of taxol. This invention also relates methods of producing taxol and related compounds from cultured *Taxus* stems and from *Taxus* roots, using hydroponics or tissue cultures.

Taxol has properties that provide significant clinical benefits. Taxol is the only plant product known to promote the assembly of microtubules and inhibit the tubulin disassembly process. Taxol and taxol-related compounds show positive activity in cancer model assays and microtubule stabilizing assays. Taxol appears to be the prototype of a new class of powerful cancer chemotherapeutic agents (Suffness & Cordell, 1985).

Taxol is a potent inhibitor of cell replication that acts as an antimitotic agent, blocking cells in the G2/M phase of the cell (Schiff & Horowitz, 1980; Horowitz *et al.*, 1986). It is unique in its ability to bind stoichiometrically to polymerized tubulin both *in vitro* and *in vivo*, and to induce the assembly of tubulin to form calcium-stable aggregated microtubule structures. These structures resist depolymerization by dilution, calcium ions, cold and a number of microtubule-disrupting drugs. Microtubules assemble tubulin in the absence of

exogenous guanosine 5'-triphosphate or microtubule-associated proteins at low temperatures (Schiff & Horowitz, 1980; Parness& and Horowitz, 1981; Howard & Timasheff, 1988).

Taxol exhibits activity against the KB cancer cell line used to screen for potential chemotherapeutic agents in tissue culture. During preclinical evaluation studies, taxol has been shown to have activity against both murine solid tumor lines and leukemic cell lines (Wani *et al.*, 1971; Suffness & Cordell, 1985; Donehower *et al.*, 1987). Taxol has been shown to be active in the OVCAR-3 human ovarian carcinoma xenograft model and to have promise in the treatment of advanced, progressive, and drug-refractory ovarian cancer (McGuire *et al.*, 1989; Thigpen, *et al.*, 1990; Einzig *et al.*, 1991; Markman, 1991).

Taxol has been through Phase III trials, and has been approved as a drug in the treatment of refractory ovarian cancer by the U.S. Food and Drug Administration. Striking effects for taxol also have been reported in the treatment of metastatic breast cancer (Holmes *et al.*, 1991), as has significant activity in the treatment of non-small cell lung cancer (Ettinger, 1992).

Taxol (NSC-125973) (Clinical Brochure; Taxol, NSC-125973 Available from Drug Regulatory Affairs Branch, CTEP, National Cancer Institute, Bethesda, MD 20892) is a cytotoxic diterpene with a complex chemistry, initially isolated from the stem bark of the western yew, *Taxus brevifolia* Nutt., by Wani and co-workers in 1969, who published the structure of the molecule in 1971. In addition to *Taxus brevifolia*, taxol has also been isolated from other species of the genus *Taxus*, including *T. baccata*, *T. canadensis*, *T. cuspidata*, and *T.* x *media* (Wani *et al.*, 1971; Witherup *et al.*, 1990; Vidensek *et al.*, 1990), and from parts of the plant including needles, young stems, woody stems, wood, bark, and roots (Miller *et al.*, 1981; Witherup *et al.*, 1990; Vidensek *et al.*, 1990; Strobel *et al.*, 1992, and Wickremesinhe, 1992).

In addition to taxol, many other closely related cytotoxic taxane derivatives have been isolated from the genus *Taxus* (McLaughlin *et al.*, 1981; Miller *et al.*, 1981; Kingston *et al.*, 1982; Huang *et al.*, 1986). Over one hundred compounds with the taxane skeleton, the basic back-bone of taxol, and related taxane diterpenoids have been isolated from various *Taxus* species (Kingston, 1991). Taxol and related diterpenoids containing the taxane ring have not been reported in any genus other than *Taxus (Jaziri et al.*, 1991).

Initially, sources pursued for production of taxol and its precursors were the bark and other above-ground parts of the *Taxus* tree. This was a reasonable strategy because those parts are the easiest to obtain.

Taxus needles have drawn attention as a stable source of taxol and its baccatin precursors (Holton, 1992 — Second Workshop On *Taxus* And Taxol; DeFuria, 1993 — International Yew Resources Conference).

Only limited explorations were made into other parts of the plant as taxol production sources, and they have not been promising. For example, two publications on the analysis and content of taxol in *Taxus* roots do not teach or suggest that roots would be a profitable source, because they indicate that the levels of taxol extracted from roots are only between one third to one fourth the units per volume as compared to levels extracted from the bark, the initial source of taxol (Vidensek *et al.*, 1990; Strobel *et al.*, 1992). Both of these reports are limited to *T. brevifolia*.

Table 1. Comparison of taxol levels found in different plant tissues of the genus *Taxus*, values are expressed as percentages of the dry weight.

	T. baccata "Repandens"	T. brevifolia	T. canadensis	T. cuspidata "Capitata"	T. media "Densiformis"	T. media "Hicksii"
Bark		0.015[a]				
Stem						
mature	0.001[a]	0.0017[b]	0.002[a]	0.004[a]	0.003[a]	0.010[a]
	0.001[b]			0.0052[c]		0.002[b]
						0.0061[c]
immature	0.0006[b]	0.0012[b]		0.0006[b]	0.0164[c]	
	0.0034[c]	0.0040[c]		0.0110[c]		0.009[b]
						0.0098[c]
Needles	0.003[a]	0.006[a]	0.009[a]	0.008[a]	0.002[a]	0.005[a]
	0.003[b]	0.0015[b]	0.006[d]	0.0183[c]	0.0145[c]	0.002[b]
	0.0016[c]	0.0076[c]				0.0124[c]
Roots		0.004[b]		0.0832[a]		0.0455[f]
Callus				0.020[e]		0.0131[f]

[a] Witherup et al., 1990
[b] Vidensek *et al.*, 1990
[c] Wickremesinhe, 1992
[d] Zamir *et. al.*, 1992
[e] Fett-Neto *et al.*, 1992
[f] present invention

****

Although Phase I and Phase II clinical studies have been conducted with taxol, the limited supply of taxol has precluded extensive clinical studies. Because there is a strong possibility that taxol will show significant activity against a wide range of cancers (Second National Cancer Institute Workshop on Taxol and *Taxus*, 1992), it is crucial to make available adequate supplies for both old and new clinical uses, and to conduct the trials leading up to those uses.

Unfortunately, producing taxol is not easy and depends on natural sources which are difficult to

replace. As mentioned previously, Taxol has been routinely extracted from the dried stem bark of *T. brevifolia*. In addition taxol has also been isolated from several other species of yew (genus *Taxus*, family Taxaceae), including *T. baccata*, *T. cuspidata*, *T. canadensis* and *T. media cvs*. Densiformis and Hicksii (Wani *et al.*, 1971; Vidensek *et al.*, 1990; Witherup *et al.*, 1990; Wickremesinhe, 1992). In Table 1 there is a summary of reported values contained in the literature of taxol found in various tissues of plants of the genus *Taxus*. With the exception of the present invention, roots showed lower levels than bark.

Although taxol has been extracted from a natural source, extraction has been extremely painstaking and, because the compounds of interest are found at extremely low concentrations in plants (0.02% dry weight basis), is associated with yields that are grossly inadequate for both present and anticipated needs. Furthermore initial extracts are contaminated with pigments, highly hydroponic cuticular substances, waxes and the like, which must be removed.

Based on the current bark-extraction procedures, 7,200 kg of bark are needed to produce one kilogram of taxol, and the projected target for 1993 is 230 kilograms (Stull, 1992). As of July 1992, taxol has been made available to more than 1,700 patients with refractory ovarian cancer (Arbuck, 1992). As more applications for taxol are revealed, the amounts of bark needed to produce taxol will increase, causing serious environmental concerns, as well as concerns about adequate and long-term supplies.

Cultivation of *Taxus* plants (yews) is unlikely to solve immediate taxol production needs. Plants of the *Taxus* genus in general have very slow growth rates and long propagation periods. This makes it difficult to implement breeding programs aimed at screening for species producing an abundant supply of these compounds. Due to the very slow growth of all yews and the low yields of taxol obtained, there is widespread concern

that alternate routes are needed to maintain an adequate and continuous supply of this drug.

Chemists all over the world have been involved in an attempt to achieve total synthesis of taxol (Gueritte-Voegelein *et al.*, 1987), but these efforts have only met with limited success. This is mainly because the precursors have not been synthesized, so a natural source is still required to start the synthetic process.

The partial synthesis of taxol starting from baccatin III has been reported (Mangatal *et al.*, 1989; Denis *et al.*, 1990; Ojima *et al.*, 1991). Baccatin III is a natural precursor of taxol and can be extracted from the needles of *T. baccata* (Chauviere, 1981; Denis *et al.*, 1988) and other *Taxus* species (Witherup *et al.*, 1990).

Even if some of the various strategies employed produce taxol, most have little practical value because they are unlikely to furnish taxol and taxol-related compounds in more than trace amounts (Denis *et al.*, 1988).

Cell cultures of *Taxus* tissues are a possible source of taxol. But long term, stable cultures have not been produced. Problems with the approach that would have had to be overcome, if roots were to be used to initiate cultures, included the fact that not all types of roots adapt to grow *in vitro* in aseptic cultures. In particular, it was not expected that *Taxus* roots would grow in culture because the *Taxus* plants grow slowly in soil, making it unlikely that culture conditions would be developed that would allow the roots to grow at an optimum rate for stable culture maintenance.

Even if it had been feasible to initiate long-term, stable cultures, production of a compound which the roots produce when grown in soil was not predictable, since those skilled in the field know well that cells do not necessarily perform the same functions in culture as they do in natural growth conditions. Thus, cells tend to undergo modifications in culture, and these modifications vary according to culture conditions. Cells disrupted from their in vivo structural architecture, do not

necessarily behave in their disorganized state, as they did in their organized state. Generally, disorganized cell cultures are unstable with regard to compounds they produced in the organized state.

Despite these problems and limitations, aseptic root cultures have been successfully established from many plants, and pharmaceutically important compounds were produced by these root cultures, albeit, in most cases at levels lower or comparable to the intact-above ground plant parts (Ishimaru *et al.*, 1990; Jha *et al.*, 1991; Sen *et al.*, 1991; Ishimaru *et al.*, 1992) For some of the plants used for root culture, the roots themselves were used for medicinal purposes, such as Sanguisorba, suggesting that the compounds of interest were present in the roots. For other compounds derived from other parts of a plant, root cultures would not be a logical source. Root cultures have been attempted for herbaceous, or "soft plants," not generally for ornamental or "woody" plants, such as *Taxus*.

There is an immediate, critical need to develop rapidly reproducible, continuously available, renewable sources of taxol and taxol-related compounds, suitable for large scale production. Because yew trees grow very slowly, planting more yew trees is not a promising solution. Chemical synthesis has not been successful and partial synthesis requires baccatin III as a starting material which must be extracted from plant material and is also found in low levels in plant tissues. Taxane ring-containing alkaloid compounds have been obtained from cell cultures of *T. brevifolia*, however, there have been problems using this approach.

The methodology described in the present invention presents a solution to the problem of taxol supply using cultured, *Taxus* tissues to produce, even overproduce, taxol, and related taxanes, in larger quantities than are possible with current taxol-production methods. An aspect of the invention is to use *Taxus* roots, despite reports on the analysis and content of taxol in *Taxus* roots that

teach away from the use of roots as a taxol source because of relatively low yield compared to bark.

Another aspect of the invention is growth of roots in hydroponic cultures. Although this method of growing plants has been established for many economically important plants for commercial greenhouse production of economically important fruits, vegetables, and ornamental plants (Moss, 1984; Adler & Wilcox, 1987; Hicklenton *et al.*, 1987; Knight & Mitchell, 1987; Kratky *et al.*, 1988; Lardizabal & Thompson, 1988; Mortley *et al.*, 1991; Brentlinger, 1992), it has not been proposed for roots *per se*. This is because roots generally are not considered when the above-ground part of the plant is the initial focal area, as is the case with *Taxus* plants. Also, hydroponic culturing is expensive, requiring special media and apparatus, so this would not be a method pursued in preference to going out and chopping bark off *Taxus* trees.

An advantage of the present invention is its eliminating the present dependence of taxol availability on *Taxus* trees in the wild. By the same token, the present invention facilitates conservation of yew trees, a large number of which need to be harvested in order to isolate the kilogram quantities presently needed for clinical use. Other advantages include increased availability and reliability of production of taxol and related compounds for the benefit of cancer patients.

SUMMARY OF THE INVENTION

Successful culture of tissues from *Taxus* plants has produced unexpectedly high yields of taxol and taxol related compounds, and more purified compounds. "Culture" includes in vitro tissue culture of stems and roots, and hydroponic culture of roots. Stems and roots are derived from natural plants, and roots may be derived from callus cultures.

Taxol is a cytotoxic diterpene with demonstrated antineoplastic activity against both solid tumor and leukemic cell lines, and activity as a chemotherapeutic agent in clinical trials. Taxol is the only plant

product presently known to promote the assembly of microtubules and to inhibit the tubulin disassembly process. This mechanism suggests that taxol is a prototype of a new class of chemotherapeutic agents that attack cancer growth in this fashion. For purposes of the invention disclosed herein, "taxol" is generally meant to include compounds which are structurally related to taxol and are defined to include precursors of taxol in biochemical pathways which are capable of producing taxol, to intermediates of those pathways, and to derivatives and secondary metabolites of taxol (10-deacetyl baccatin III, 7-epi-10-deacetyl baccatin III, baccatin III, 7-epi baccatin III, 9-dihydro-13-acetyl baccatin III, cephalomannine, 10-deacetyl taxol, 7-epi10-deacetyl taxol, 7-epi taxol) and other known and unknown natural derivatives of these compounds and related taxanes resulting in taxol production and/or the production of novel antitumor/antiviral compounds.

"Taxol-like activity" refers to compositions which show positive results in at least one test predictive of effective chemotherapy: a microtubule stabilizing assay, cancer model assay, or clinical trial.

Successful tissue culture methods have been developed which result in long-term, stable cell lines derived from *Taxus* explants. Stable cultures are cultures that maintain a taxol production level after repeated subcultures. Long-term cultures re those that remain stable for at lease one year, preferably two or more years. Cultures produced by methods of the present invention have remained stable for up to seven years. Some of the cell lines are habituated, that is, they do not require plant hormones as supplements to the nutrient culture medium for long-term maintenance. The cell lines established by use of the methods of the present invention are capable of producing taxol, and taxol-related compounds. These cell lines offer a rapidly reproducible, continuously-available source for the production of taxol and taxol-related compounds. This

production may be scaled to commercial levels by use of the cell lines in bioreactors. In addition to obtaining the same compositions as presently directly extracted from yew trees, new compositions exhibiting taxol-like activity, have been purified from extracts of the *Taxus* cell lines, offering new horizons for chemotherapeutic agent development.

By combining steps not previously combined to produce *Taxus* cultures and by using new methods and compositions, successful long-term growth has been achieved, and distinct cell lines produced. A method for establishing a culture of cells derived from the genus *Taxus* has been successful in producing cell lines which secrete taxol and taxol-related compounds into the culture environment. The steps of the in *in vitro* tissue culture method include the following:

(1) Selecting an appropriate tissue source for an explant which will be used to initiate the culture

The general tissue source is plants of the genus *Taxus*. Various species from which taxol may be isolated include *T. brevifolia* Nutt, *T. baccata, T. cuspidata, T. canadensis cv.* Capitata, *T. media cv.* Densiformis, and *T. media cv.* Hicksii. Preferred sources for explants used to produce taxol- expressing cultures are those plants from which the amount of taxol that may be extracted directly is high when compared with amounts extracted from other species by comparable methods from comparable amounts of starting material. By that criterion, *T. media*, more particularly *T. media cv.* Hicksii, for example, is a more preferred source than *T. brevifolia* when stems are used as a tissue explant to initiate the cultures. Tissue explants are a portion of tissue selected from a plant and transferred into appropriate tissue culture vessels such as Petri plates or Magenta (a square dish). The explant is then cultured as described herein.

Bark had been the usual source of taxol, and needles and roots also are capable of expressing taxol. It was found, however, that a preferred tissue source of the explant is a stem, more particularly a young, fresh, succulent stem. Young stems are those that appear at the beginning of a new growth season for the plant. Immature stem sections of *Taxus media cv*. Hicksii are particularly preferred because stems of *Taxus media* plants contain higher levels of taxol than *Taxus brevifolia* and other *Taxus* species. Explants of about 0.3-0.5 mm in diameter are suitable. Root explants are more preferred in the context of the present invention.

(2) Culturing the explant in callus-inducing medium to form callus

One of the combinations of factors leading to successful *Taxus* cultures includes use of a callus-inducing medium in which to grow the explant.

To form an explant, the tissue obtained from the plant is processed by surface sterilization and sectioned under aseptic conditions. Sectioning is accomplished by cutting a stem into pieces of approximately 6-8 mm in length, from immature stems that are approximately 3-5 mm in diameter, and transferring the sections to tissue culture vessels, generally, petri dishes, with callus-inducing nutrient media. This is medium which is capable of initiating and promoting the formation of callus growth from the explant. A preferred media is Gamborg's B5 media (Gamborg *et al.*, 1968) supplemented with plant hormones such as 2,4-D and kinetin either alone or in combination. Murashige/Skoog medium (Murashige & Skoog, 1962) also is suitable for this purpose and may be supplemented. Gelling agents such as Gelrite (Scott Laboratories, West Warwick, R.I.) or agar are mixed with the nutrient media to form a solid support in the culture vessel for maintenance and growth of the explant.

(3) Subculturing selected sections of the callus at intervals which promote subclone growth

Callus formation is determined when globular aggregates of undifferentiated cells are observed. This

generally occurs on 90% of the explants within about 2-4 weeks. Subculturing initially is performed at 4-6 week intervals, but may be altered as a function of culture growth.

(4) Selecting a subclone to develop a cell line

An important step in creating cell lines which are capable of producing taxol is choosing the callus sample to select for subculturing to develop a subclone of the callus. Physical selection criteria have been developed as an aspect of the present invention. Callus tissue with red coloring should not be selected because the red color indicates the presence of phenolic exudates which inhibit callus growth. Callus sections exhibiting a yellow color are preferably selected for subculturing. Cultures growing well in the dark will be pale yellow to light brown.

Friability is another selection criterion. This is determined by observing a crumbly, fragile texture of the callus. Another factor which indicates samples that are likely to result in successful cell lines for purposes of the present invention is relative growth rate of the callus cells. Initially, all callus are slow growing, taking about 2-4 months to double. Preferable doubling times are from about 5-16 days. Subculturing was performed at intervals of about 4-6 weeks, although the interval may be shorter or longer depending on the health of the callus, as indicated by color, and by the size of the callus after growth.

By serial subculturing of callus samples following these criteria, relatively homogeneous subclones with regard to these criteria, develop. Although the time between an explant being placed in culture and the appearance of subclones varies, some of the intervals are measured in years.

(5) Culturing the cell line on a solid support in maintenance medium

Two other important factors for successful *Taxus* culture are a solid support in the form of a membrane raft, and maintenance media for long-term culture growth.

The optimum culture conditions for the selected subclones then are determined by a multivariate analysis of factors which are likely to effect culture growth: presence or absence of light, temperature, pH, medium supplements including plant hormones and sugars. Sucrose has been used by those of skill in the art as a sugar in nutrient medium for *Taxus* cultures. Unexpectedly, fructose and glucose are used for the present invention to enhance culture growth, with or without sucrose. Also surprisingly, higher concentrations than previously recommended for sucrose if used as a single sugar were found to enhance cultures. High concentrations of either glucose or fructose used singly also gave excellent results. None of these sugar protocols had been reported for *Taxus*. Casein Hydrosylate is beneficial to support optimal growth.

Optimum culture maintenance factors included use of a membrane raft to support the cells. This structure facilitated subculturing, collection of extracts, and formation of cell suspensions. After stable culture growth is achieved, the cell lines are kept in maintenance medium. For some of the cell lines, habituation occurs; that is, the cultures are maintained without the need for hormonal supplements.

In addition to callus cell lines, cell suspension cultures are induced from well established stable callus cultures as described above by transferring an inoculum of cells (2-3 grams) of friable callus clumps into liquid medium contained in Erlenmeyer flasks (generally 250 ml flasks containing 100 ml of medium). Flasks are placed on a rotary shaker known to those of skill in the art and operated at about 125 rpm.

An important use of *Taxus* cell lines within the present invention is to produce taxol and taxol-related compounds. Callus and suspension cell lines of *Taxus* developed by the methods of the present invention produce taxol, precursors and intermediates of a biochemical pathway resulting in taxol production, including cephalomannine and baccatin, and active derivatives of

taxol. Both biotic and abiotic elicitors, precursor feeding and other elicitation techniques are useful to enhance secondary metabolite production. Taxol, and taxol-related compounds, are extracted from callus, suspension cultures and culture medium. An "active" composition is one that is capable of giving positive results in a microtubule stabilizing assay, a cancer model assay, a clinical trial, or any combination of these tests. These cell lines are a continuous source because they can be maintained indefinitely, some have now been maintained for 5-7 years, under aseptic conditions and retain their ability to produce taxol, active derivatives and precursors of taxol. They are likely to grow well in large scale bioreactors and the cells can be cryopreserved. Taxol production ability is retained after cryopreservation or storage at 4-10°C.

Cell lines are suitable as sources to grow in bioreactors to produce large quantities of taxol, and taxol-related compounds. Some of these compositions are found in the culture medium and may be obtained by collecting the medium. Utilizing different bioreactor designs, the secondary metabolites found in solution may be easily removed from the solution utilizing exchange resins which extract only the desired secondary metabolites from the solution and not the media supplements required for culture growth. Other compositions are produced and retained intracellularly and must be extracted from the cells.

The growth of *Taxus*-derived cell lines in bioreactors enables the production of much larger quantities than are presently feasible. This process not only serves as a renewable resource of chemotherapeutic agents but also saves the large number of trees that are presently being harvested to isolate this compound.

Taxol is found in the roots of all the different *Taxus* species and cultivars cultured according to the present invention and evaluated. The levels range from amounts comparable to the levels found in the above ground parts to amounts 2- to 4-fold higher. Objectives

of the present invention include growing *Taxus* roots. Successful methods utilizing *Taxus* roots - including roots cultured hydroponically, *in vitro* cultured roots, adventitiously produced roots, roots from callus derived from stems or needles and *Agrobacterium* transformed root cultures have been developed which result in long-term, stable production of taxol and related taxanes.

The roots established by use of the methods of the present invention are capable of producing taxol, and taxol-related compounds. These roots offer a rapidly reproducible, continuously-available source for the production of taxol and taxol-related compounds. This production may be scaled to commercial levels. In addition to obtaining the same compositions as presently directly extracted from yew trees, new compositions exhibiting taxol-like activity, have been purified from extracts of the *Taxus* roots, offering new horizons for chemotherapeutic agent development.

Taxus roots, including roots cultured hydroponically, normal roots cultured *in vitro*, adventitiously produced roots, roots from callus produced from stems and needles, and *Agrobacterium* transformed callus or root cultures, have the potential to produce an abundant supply of taxol and of biologically active compounds and precursors. In addition to providing a rapidly available and renewable source of taxol there are many other potential benefits.

Advantages realized in the use of hydroponically produced roots for the production of taxol and related compounds include: (1) genetically stable roots can easily be produced from all *Taxus* species and cultivars available, within a short period of time, thereby expediting the search for a high producing source of taxol along with novel taxanes and related compounds, (2) the ability to control the supply of secondary metabolites is independent of weather, disease, and politics to meet market demand, (3) strain improvement programs analogous to those for microbial systems can be initiated, (4) novel products such as taxol and its

derivatives can readily be obtained from the new methods, whereas, this can not be done with existing methods, (5) extracts from roots are very clean, thereby, overcoming the need for extensive purification which is necessary from extracts obtained from leaves, stems or bark which contain chlorophyll and other pigments which make extraction and purification difficult, and (6) roots from plants grown hydroponically grow much more rapidly than the top portion of the plant, and may be maintained to grow continuously, even though the plant undergoes a dormant period.

Production and evaluation of taxol and related compounds in hydroponically grown roots or aseptically grown roots from the genus *Taxus*, is a novel accomplishment. In addition, the extraction protocols to purify taxol and related taxanes from *Taxus* bark are very complex, and extraction from stems and needles makes the process even more complicated due to the presence of chlorophyll, other pigments, waxes and unknown hydrophobic compounds. In contrast, taxol extracts from stem tissues culture or root cultures, including those grown hydroponically, are relatively clean and require less purification to be usable.

As an example of the comparative purity of root cultures, when stems, needles or bark are extracted with methylene chloride, there is a dark brown or green viscous extract resulting which does not occur with roots. Extracts from stems, needles or bark can be directly analyzed by HPLC, however, the presence of highly hydrophobic compounds clog the column resulting in a shift in the baseline resulting in a less pure sample. In addition the initial peak coming off the HPLC is broadened showing additional contaminates from water soluble pigments thereby causing problems with purification of baccatins. In order to avoid this situation it is necessary to add an initial purification step using hexane partitioning prior to methylene chloride extraction to remove hydrophobic compounds and a Sepak C18 cartridge to remove water soluble compounds.

This is not necessary with root extracts since they contain no pigments and few hydrophobic compounds.

Roots cultured in accordance with the present invention are a continuous source of taxol because they can be maintained indefinitely, under aseptic conditions and retain their ability to produce taxol, active derivatives and precursors of taxol. They grow well in large scale bioreactors and can be cryopreserved. Taxol production ability is retained after cryopreservation or storage at 4-10°C.

Roots are suitable as sources to grow in bioreactors to produce large quantities of taxol, and taxol-related compounds. Some of these compositions are found in the culture medium and may be obtained by collecting the medium. Utilizing different bioreactor designs, the secondary metabolites found in solution may be easily removed from the solution utilizing exchange resins which extract only the desired secondary metabolites from the solution and not the media supplements required for culture growth. Other compositions are produced and retained intracellularly and must be extracted from the roots. Compounds that permeabilize membranes facilitate taxol extraction.

The growth of *Taxus*-derived roots in bioreactors enables the production of much larger quantities than are presently feasible. This process not only serves as a renewable resource of chemotherapeutic agents but also saves the large number of trees that are presently being harvested to isolate this compound.

Root extracts require fewer purification steps and generally result in more purified compositions than are obtainable from other parts of the plant. Roots contain fewer contaminants such as pigment and wax than are present in the aboveground portion of the plant. Roots therefore allow for better resolution of more taxanes by chromatography. The upper portion of the plant is contaminated with waxes, pigments such as chlorophyll and other interfering compounds which are very abundant in the aboveground portion of the plant. In fact even after

purifying materials from the upper portion of the plant, there are still problems with these samples clogging HPLC columns. The actual profiles from HPLC corresponding to different taxanes are purer when roots are the source material. The more purification steps required, the more loss resulting in lower recovery, thereby, resulting in higher costs due to labor, solvents, and the like.

Roots have been used as a source of secondary metabolites in plants, but they have not been employed from *Taxus* plants and have not been recommended as a source of compounds from woody plants. An advantage of using taxus plant roots is that *Taxus* plants are slow growing and require special conditions for proper growth. This problem along with the high cost and increasing demand for taxol and related taxanes, makes the use of roots from hydroponically grown plants very attractive. It is likely that taxol is synthesized in the roots and is transported to the rest of the plant where it accumulates. This makes the roots ideal for biosynthetic studies of taxol related compounds because all of the machinery (enzymes) is present, therefore roots are a good source of precursors to be used in partial synthesis of taxol and other novel antitumor compounds. Moreover, root culture readily lends itself to elicitation.

Organized tissue such as roots have less of a problem than cell culture because they are more homogeneous than cell cultures which are typically heterogeneous. Cell suspensions have an inherent problem with an unknown compound which cochromatographs with taxol, which is not a problem with root culture.

In callus derived roots, that is, not *Agrobacterim* infected, there is a problem with somoclonal variation from natural roots. To overcome slow growth of natural roots, making it difficult to start woody plant roots in tissue culture, somoclonal variants which have been observed to grow relatively fast may be used.

While there is an extensive body of information on the organism *Agrobacterium rhizogenes*, little is known

about its interaction with plant cells. There are many cases where specific bacterial strains will infect a plant, but there are numerous plants which can not be infected. More knowledge is necessary on how the plant interacts with the bacterium before the process becomes standard. The most important factor effecting the ability of a bacterium to infect a host is the wound response. Plants and tissues differ in the wound response and only plants with a pronounced wound response can be readily infected. Woody plants such as *Taxus* are not readily wounded, therefore are difficult to infect. (Potrykus, 1991).

The methods and compositions of the present invention facilitate new screening tests to determine which species and varieties of *Taxus* are amenable to long-term, stable culture, or hydroponic culture, and which sources are likely to yield taxol and taxol-related compounds. In addition to rapidly reproducible, continuously available, accessible and renewable sources of these compositions provided, keys to accessing the unknown variations in taxol-related compounds, some variations not obtainable from the natural source, also are provided by the present invention. These variations may be even more potent chemotherapeutic agents than have been reported.

Previously unknown components of the *Taxus* cultured cell extracts with taxol-like activity have been eluted as peaks by HPLC (high pressure liquid chromatography). These peaks contain materials which are positive in microtubule stabilizing assays. The production of compositions with taxol-like activity from cultures, compositions that may not be present in natural sources, expands the horizons for development of chemotherapy agents. Also, because cell lines differ in their HPLC profiles related to these compositions, cell culture appears to be a useful source of new treatment agents.

The cell lines differ in the amount and nature of taxol and taxol-related compounds that they produce in culture under a defined set of environmental conditions.

Table V illustrates differences among some of the cell lines with regard to the range of percent of taxol produced. Components of peaks are taxol-like if they are capable of stabilizing microtubules and/or of giving positive results in cancer model systems, for example, those based on the toxicity of a test agent to cells.

Another aspect of the present invention is to produce new *Taxus* cell lines by inducing mutations. Mutations of selected cell lines are induced by irradiation treatment and chemical mutagens. The callus and subclone selection criteria and methods to induce callus and maintain cell lines as described herein, are used to produce cell lines from the mutated sources. Ultraviolet light, in particular UV-A, and chemical mutagens such as ethyl methane sulphone, are suitable mutagens. Selection for naturally occurring mutations due to somoclonal variation, is also useful.

Another aspect of the present invention is the regeneration of *Taxus* plants from *in vitro* cultures. For the regeneration of taxol-producing plants, callus cultured on a high auxin:cytokinin ratio under a 16 hour regime was induced to undergo organogenesis by serially decreasing the auxin : cytokinin ratio [to 2IP (2-isopentenyladenine) 2 - 10 mg/l in combination with IBA (indole-3-butyric acid) 0.1 - 2.0 mg/l, for example]. Shoots produced by these callus were then excised aseptically (approximately at the stage of 1 cm shoots) and placed on Gamborg's B-5 basal medium supplemented with 20 g/l sucrose to induce root formation. Well rooted shoots are then transferred onto sterile Metro-mix 200 potting medium, acclimated and transferred to the greenhouse to develop into plants. Regeneration of plants from cultures with increased production of taxol provides plant sources that are capable of producing greater amounts of taxol and taxol-related compounds than natural plants can produce.

Roots from a wide range of *Taxus* species and cultivars grown under optimized conditions described herein are elicited both *in vivo* and *in vitro* with biotic

(ie. fungal extracts) and abiotic elicitors (i.e. heavy metals), plant growth regulators, precursors and/or intermediates in the taxol biochemical pathway (i.e. acetate, mevalonic acid) resulting in an increase taxol and related taxanes and/or novel antitumor compounds.

A major benefit of obtaining roots from hydroponically grown plants is that they are genetically stable, yielding uniform results overcoming a typical problem when dealing with roots in tissue culture. Because elicitation of taxol and related taxanes was successful, it appeared likely that genes involved in the biosynthesis of taxol and related taxanes were stimulated. Based on this knowledge, cDNA libraries were constructed and through differential screening of these libraries, several putative genes involved in taxol biosynthesis were identified. These genes are likely to be overexpressed in tobacco plants and/or bacterial systems which could potentially be used to overproduce taxol and related compounds. Both biotic and abiotic elicitors, precursor feeding and other elicitation techniques are useful to enhance secondary metabolite production.

DEFINITIONS

An "active" composition: is one that is capable of giving positive results in a microtubule stabilizing assay, a cancer model assay, a clinical trial, or any combination of these tests.

Bioreactors: apparatus for growing roots on a large scale.

Airlift: Kontes (Ctyolift airlift bioreactor)
Braun Biotech (Biostat air lift bioreactor)
LH Fermentation (500 series)

Stirred: New Brunswick (CelliGen & BioFlo)
LH Fermentation

Perfusion: New Brunswick

Membrane-flow: nutrient medium flows over a membrane which supports the roots, the medium is recirculated. Other novel designs are suitable.

Callus: an aggregate of dedifferentiated cells.

Cancer model systems: *in vitro* or *in vivo* systems wherein a result is produced by test agents, said result determined to be predictive of clinical effects of the agent.

Cell Culture: composition of living cells maintained *in vitro*.

Cell Line: group of cells *in vitro* for which growth characteristics have become stable under a defined set of environmental conditions, and which is differentiated from other cell lines by these characteristics.

Cell Suspension: composition of living cells that are not attached to a solid substrate but rather are suspended in a liquid.

Clumps: globular or nodular aggregates of cells observable on the surface of a callus.

Conditioned medium: medium in which cultured cells secreted into the medium various metabolites and other components of cell growth and division.

Culture growth rate: used herein to mean the rate at which cells in culture double by mitosis; doubling rate.

Dormant Plant: external factors are not promoting growth of the portion of a plant above ground.

Elicitation techniques: techniques applied to a cell culture which increase the yield of taxol or taxol-related compounds that are produced by the culture; the mechanism of this empiric effect may be immunological, that is, the cells may mount a defense against foreign agents by producing taxol.

Abiotic: examples include salts of heavy metals,vanadate; lead and mercury.

Biotic: examples include autoclaved fungal cell wall fragments and spores.

Friability: fragile, crumbly tissue consistency.

Habituated cell line: cells *in vitro* which are maintained by subculture and do not require nutrient medium supplemented with hormones for maintenance; these cultures are usually characterized by relatively rapid growth rates.

Habituation: condition wherein a cell line is capable of being maintained by serial subculture without the use of hormones as supplements to the nutrient medium in which the cells are maintained.

Hairy root disease: A disease of broadleaved plants in which there is a proliferation of root-like tissue from a plant part. This is a tumorous state similar to the crown-gall. Hairy root disease is induced by the bacterium *Agrobacterium rhizogenes* containing an Ri plasmid.

Hydroponics: Water culture of plants. A plant culturing system where roots are developed without soil.

In vitro: literally "in glass", an artificial environment which mimics the *in vivo* conditions for maintenance and, generally, growth and reproduction of biological materials.

In vivo: literally "in life". Pertaining to a biological reaction that takes place in a living cell or organism.

Medium: a nutrient composition for the growth of living material such as cells, tissues, embryos and the like.

Nutrient film technique (NFT): A method for growing plants that ensures an adequate supply of both oxygen and water to the roots. Plant roots need both water and oxygen for satisfactory plant growth. Roots in soil are deprived of oxygen if there is abundant water. Conversely, good aeration is usually associated with lack of water. In NFT, plants develop their root systems in a very shallow stream of nutrient solution. The solution depth is adjusted so that the lower part of the developing root mat grows wholly in solution. The upper part projects just above the surface, but remains covered by a liquid film. This exposed upper part allows for good aeration. The root mats produced by each plant are extensive and interwinded; this condition allows the plants to become self-supporting.

Other standard or novel hydroponic systems are also suitable for the present invention.

Retention time: time at which a given compound will elute off a chromatographic column counting from the time at which the sample was introduced onto the column.

Secondary metabolite: a metabolite that is not part of the primary metabolic network of the cells. Secondary metabolites are usually present only in cells that specialize in some way (e.g. defense).

Shaker flasks: any container that could be used to culture cells on a rotary shaker, e.g. Erlenmeyer flasks.

Sparging: forcing (bubbling) air through a liquid medium.

Stable culture growth: condition of cell culture growth wherein doubling times of the culture have reached an equilibrium under a defined set of culture conditions, and taxol production levels are maintained.

Subclone: a culture derived from a sample of cells from a parent culture.

Subculture: a cell culture composition derived from a sample of cultured cells, said sample being transferred to a different tissue culture vessel than that containing the culture of cells from which the sample was obtained.

Tissue culture: composition of living tissue maintained *in vitro*; the terms"cell culture" and "tissue culture" are often used interchangeably, as they will be herein to refer to any composition where a plurality of cells are maintained *in vitro*.

Agrobacterium rhizogenes A species of Gram-negative, rod-shaped bacteria closely related to *Agrobacterium tumefaciens*. *Agrobacterium rhizogenes* often harbors large plasmids, called Ri plasmids, which are closely related to Ti plasmids. The combination of *A. rhizogenes* and an Ri plasmid can cause a tumorous growth known as hairy root disease in certain types of plants.

BRIEF DESCRIPTION OF THE DRAWINGS

Other objects and advantages of the invention will become apparent upon reading the following detailed description and upon reference to the drawings in which:

FIGURE 1. HPLC profile of taxane standards separated on a Curosil column: 1. Taxol; 2. 7-epi-10-deacetyl taxol; 3. Cephalomannine; 4. 10-deacetyl taxol; 5. Bacctin V; 6. 9-dihydro-13-acetyl baccatin III; 7. Baccatin III; 8. 7-epi-10-deacetyl baccatin III; 9. 10-deacetyl baccatin III.

FIGURE 2. HPLC profile of a stem culture of *T. media cv.* Hickii (CR-1 callus)

FIGURE 3. HPLC profile of a root culture of *T. media cv.* Hickii

FIGURE 4. HPLC profile of a root culture of *T. canadensis*

FIGURE 5. HPLC profile of a root culture of *T. cuspidata*.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Using the methods of the present invention, success in producing stable, long-term *Taxus* cell lines has been achieved, and various forms of *Taxus* roots have been grown hydroponically. The following combinations of steps are novel for creation of Taxus cell lines.

The cultures of the present invention are induced from the *Taxus* species including: *T. brevifolia, T. canadensis, T. chinensis, T. floridana, T. globosa, T. sumatrana, T. yunnanensis and from different cultivars of T. baccata. T. cuspidata and T. media* and others from the genus *Taxus*.

Young stem explants or roots are preferred as a tissue source to induce callus. Young stems are those that appear as new growths on a *Taxus* plant; the stems generally are about 0.3-0.5 mm in diameter. Young stems of *Taxus media cv.* Hicksii are preferred because these stems are found to produce higher levels of taxol than other *Taxus* species including the stems of *Taxus brevifolia*. Although other plant parts such as roots have the ability to produce callus, young stems provided the best results on a consistent basis. Roots derived from callus, and roots from other sources, however, produce the highest yield of taxol when cultured in tissue culture or hydroponically.

Young stem explants from *Taxus* are surface sterilized and sectioned under aseptic conditions. Sections are grown on callus-inducing medium. Suitable medium includes Gamborg's B5 medium (Gamborg *et al.*, 1968) (Materials and Methods) fortified with Gamborg's B5 vitamins, casein hydrolysate (1.0 g/l), and a combination of fructose, glucose and sucrose totalling 25-40 g/l. For some cell lines, high concentrations of glucose, fructose or sucrose (40-80 g/l) is preferred to the standard 20-30 g/l of sucrose in nutrient medium. Surprisingly, glucose and/or fructose may replace sucrose. Plant hormones are used to supplement the medium as plant growth regulators for callus induction. Auxins may be used in combination with kinetin, the latter at a range of 0.1-1.0 mg/l. In determining ratios of hormones to use, a high auxin: cytokinin ratio is preferred for callus induction, for example, 1 mg/l 2,4-D to 0.2 mg/l kinetin; 5 mg/l of naphthalene acetic acid (NAA) to 1 mg kinetin. With regard to auxins, NAA (at a range of 1.0-5.0 mg/l), 2,4-D (at a range of 0.1-2.5 mg/l) or IBA (indole butyric acid, at a range of 1.0-5.0 mg/l) are suitable. In an illustrative embodiment, 1 ppm 2,4-D and 0.2 ppm kinetin are added to the cultures, alone or in combination with Gelrite or agar as gelling agents.

Explants are placed in tissue culture vessels, which can be petri dishes containing medium gelled with Gelrite, at about a 2 g/l concentration. Prior to being dispensed into sterile petri dishes, the medium may be sterilized, for example, autoclaved at 121°C for about 15 minutes.

Explants are incubated in complete dark at a temperature of about 23-25°C. The pH of the medium is preferably maintained in the range of 5.5-5.7. Some cultures may prefer to grow in the light; for example, cool, white fluorescent light for 16 hours in a 24 hour cycle.

As a general rule, induced callus is initially very slow growing and produces phenolic exudates (causing the

callus to turn red) which are detrimental to the growth of the callus, even when the callus is frequently subcultured. As described in more detail in the "Materials and Methods" section herein, subclones may be selected from the subcultures of induced callus based on physical criteria including comparative growth rates, absence of red coloring, presence of yellow coloring, and friability.

Subcultures selected by the criteria disclosed herein, are transferred to solid supports, preferably membrane rafts (Sigma Chemical Co.) on media such as that shown in Table IV. Membrane rafts were preferred for the culture of callus because this system is similar to a "membrane-flow" type bioreactor. By use of a raft, the culture medium is changed and manipulated easily, and thus can easily be adapted to large scale culture. Other reasons for using rafts are that callus tend to grow better on membrane rafts than on agar or other gelling agents, and also it is easier to harvest callus both for analysis and subculturing purposes.

Methods for culturing normal roots in tissue culture, are as follows:

Roots are produced in tissue culture by several different methods: root tips from hydroponically grown plants and adventitiously derived roots produced from stem/needle explants. This is done according to the methods of Wickremesinhe and Arteca (1993) who were successful in this approach with *Cephalotaxus harringtonia* (Japanese plumyew). Each of the explant sources are processed by surface sterilization and sectioned under aseptic conditions. The explants are then transferred to tissue culture vessels, generally, petri plates, with root-inducing media for root tip and root sections or to callus-inducing media for stem/needle explants (once rapidly growing callus was established it was transferred to root inducing media). These media are capable of initiating and promoting root callus growth from the explant. A preferred media is Gamborg's B5 media (Gamborg *et al.*, 1968) supplemented with plant

hormones such as auxins and cytokinins either alone or in combination. Other tissue culture medias such as that of Murashige and Skoog (Murashige and Skoog, 1962) and others are also suitable for this purpose and may be substituted. Gelling agents such as Gelrite (Scott Laboratories, West Warwick, R. I.) or agar are mixed with the nutrient media to form a solid support in the culture vessel for maintenance and growth of the explant.

Selected roots or callus are subcultured at intervals which promote subclone growth. Roots which are actively growing after 2-4 weeks are selected for transfer. Callus formation is determined when globular aggregates of undifferentiated cells are observed. This typically occurs on 90% of the explants within about 2-4 weeks. Subculturing was initially performed at 4-6 week intervals. The doubling is initially very slow, however, with time in culture the doubling rate of selected clones increases to 5-16 days allowing for subculture every 2-3 weeks depending on the clone. After establishing stable callus lines for each of the species/cultivars previously mentioned, callus is transferred to root inducing media where it generally takes 4-6 weeks to initiate roots.

Methods for culturing of "Hairy Roots" in tissue culture, are as follows:

Immature roots and shoots from hydroponically grown *Taxus* plants are inoculated with several strains of *Agrobacterium rhizogenes* strain 11325, 15834 and others. The cuttings are inoculated with individual *Agrobacterium* strains using standard protocols. Immature taxus roots and shoots are surface sterilized and placed on Gamborgs B5 medium. The explants are wounded aseptically with a syringe tip and inoculated with the appropriate strain. After 48 hours the explants are rinsed with sterile water and placed on fresh media containing 500 µg/ml carbenicillin. After 4-6 weeks "hairy roots" that appeared at the site of infection are excised from the stem or root and placed on Gamborg's B5 media containing carbenicillin 600 µg/ml in order to kill any residual *Agrobacterium*. The transformed roots are subcultured

every 2 weeks onto at least three subcultures with antibiotics to ensure that all the bacteria were killed. The transformed roots are transferred to shake flasks and grown for several weeks prior to transfer to commercial fermentors available. These included the air-sparged system, free stirred batch system and impeller-mixed fermentation systems ranging from 1.5 to 260 liters in capacity. The upper are preferred for commercial production.

In vitro and *in vivo* growth of roots is optimized by the following procedure:

The optimum culture conditions for the selected subclones are then determined by a multivariate analysis of factors which are likely to effect culture growth: presence or absence of light, gas composition, temperature, pH, medium supplements including plant hormones and sugars. After stable culture growth is achieved, the root cultures are kept in maintenance medium. For some of the root cultures, habituation occurs, that is, the cultures are maintained without the need for hormonal supplements.

In addition to roots grown on a solid support, root tips from established stable root cultures as described above were transferred into liquid medium contained in Erlenmeyer flasks (generally 250 ml flasks containing 100 ml of medium). Flasks are placed on a rotary shaker known to those of skill in the art and operated at about 125 rpm.

A method for culturing *Taxus* roots hydroponically, growth of normal roots *in vitro*, adventitiously produced roots, roots from callus derived from stem and needle explants and *Agrobacterium* transformed roots which produce taxol and taxol-related compounds the steps involved are as follows:

(1) <u>Appropriate tissue is selected as a cutting source and initiate rooting</u>

The general tissue source are plants of the genus *Taxus*. Various species from which taxol may be isolated include: *T. brevifolia, T. canadensis, T. chinensis, T.*

floridana, T. globosa, T. sumatrana, T. yunnanensis and from different cultivars of T. baccata. T. cuspidata and T. media and others from the genus *Taxus* were rooted. The selection criteria for the source of cuttings, species or cultivar used is based on the following properties:

a. high taxol production

b. high 9-dihydro-13-acetylbaccatin III production

c. high baccatin III production

d. high levels of other known precursors of taxol

e. high levels of unknown taxanes as determined by HPLC analysis.

As shown in FIGURES 2-5, peaks not previously identifiable (peaks A-K) appear in tissue culture extracts. Profiles differ in stem (FIGURE 2) compared to root (FIGURES 3-5) derived cultures, and among root cultures from different species. Retention times are as follows:

A=33.6
B=34.9
C=36.1
D=37.9
E=39.9
F=42.3
G=44.4
H=46.2
J=50.8
K=51.8

The cuttings are rooted in two different ways -- one in a soil mix, and the other in solution. *Taxus* plants are relatively easy to propagate from cuttings, however, methods vary. *Taxus* cuttings are highly topophytic and will maintain the growth habit they exhibit on the parent plant. In one example, cuttings were collected from September through December after a hard frost. These are roots from dormant plants, which may explain high yields of taxol from root cultures. Metabolic root activity may

be higher during plant dormancy. Cuttings taken after a frost typically root more readily than cuttings without a frost.

After cuttings are taken the needles are stripped from the lower portion of the stem. 8,000 ppm IBA-talc plus a fungicide was used for most cultivars, however, some cultivars prefer a 5,000 to 10,000 ppm IBA quick dip.

There are many types of media which can be used, but a sand or a sand/peat mix is preferred. The base of the cutting is maintained at 68° to 75°F with the tops kept cool and placed under mist to prevent desiccation. The cuttings show branched roots 8 to 9 weeks following planting (Dir and Heuser, 1987).

After this period of time the roots are washed and the plants transferred to hydroponics. An alternative to placing the cuttings into the soil is to treat the base of the cuttings as previously mentioned and place them directly into hydroponics.

One of the factors in a combination leading to optimization of root/shoot growth, together with the maximum production of taxol and related taxane production in roots, is to first maximize *Taxus* plant growth and development. This is accomplished by transferring the rooted cuttings to hydroponics: in the root zone the optimization of nutrient levels (Hoagland and Arnon, 1938), is achieved by testing combinations of CO_2/O_2; temperatures and plant growth regulators including auxins, cytokinins, gibberellins, ethylene, brassinosteroids, abscisic acid, growth retardants such as cycocel, B9 and other regulators alone or in combination. In addition, the shoots of hydroponically grown plants also require the gas composition (CO_2 enrichment), environmental conditions including light, temperature, humidity and plant growth regulators optimized. The optimal conditions for each of these parameters for roots and shoots differs with the species used and the specific taxane desired, whether roots are grown for high levels of taxol, 9-dihydro-13-acetyl

baccatin III, baccatin III, other known and unknown taxanes will dictate the optimal conditions used.

Taxol, active derivatives of taxol, cephalomannine, baccatin and other precursors and intermediates of taxol can be examined using HPLC analysis by comparing the retention times of authentic standards and plant extracts. This analysis is accomplished by extracting callus, performing HPLC analysis on the extracts, and collecting components of major peaks. The components of these peaks are tested for activity in microtubule stabilizing assays (FIGURE 5, Materials and Methods) and in *in vitro* cancer model systems disclosed herein. Cancer cell lines suitable for screening include B16 Melanoma; MX-1 mammary xenograft; P388 leukemia; KB; L1210 leukemia.

Taxol activity is also confirmed in cell lines by showing that extracts have the ability to stabilize microtubule activity in the same manner as taxol. Another means of identification of taxol is accomplished by extracting callus cell lines, purifying this extract by HPLC and identifying taxol by utilizing H^1 and 2-D high resolution nuclear magnetic resonance (NMR) analysis (Falzone *et al.*, 1992).

Different bioreactor designs are available to maximize growth rates, taxol, active derivatives of taxol, cephalomannine, baccatin and other precursors of taxol made by each of the cell lines and secreted into solution (Weathers and Giles, 1988; Payne *et al.*, 1987). Plant cells immobilized in various gels and membrane reactors have been used in bioreactors (Brodelius, 1985). Alginate, agar, agarose, kappa-carrageenan, polyacrylamide, polyurethane and various membrane configurations have been used to entrap cells (Brodelius, 1988; Smidsrod and Skjak-Braek, 1990). By optimizing environmental conditions such as light levels, gas compositions/dissolved oxygen, temperature, plant hormones, pH of the growth media, nutrients, vitamins and removal of taxol from the media, desired compound extraction may be maximized.

The levels of taxol found in the cell lines of the present invention are at least 1 to 2 fold higher than in stems, leaves and bark of *Taxus media cv.* Hicksii (Table I). These levels may be increased further. In general for a variety of tissue cultures, it has been shown that cultures producing low or undetectable levels of secondary products are elevated by a variety of means including optimization of culture conditions, precursor feeding, use of biotic (Heinstein, 1985; Fukui *et al.*, 1990), and abiotic elicitors including the use of ultra-violet irradiation (Kartusch and Mittendorfer, 1990) and heavy metal ions (Threlfall and Whitehead, 1988). The addition of precursors to cell cultures of *Tripterygium wilfordii* has been reported to increase the production of the antitumor compound tripdiolide (Misawa *et al.*, 1985). Precursor feeding, biotic and abiotic elicitors as defined herein, are suitable for use with *Taxus* culture.

In accordance with the present invention, plants may be regenerated from *Taxus* cultures to provide yet another source of taxol and, more interestingly, perhaps of the new compounds identified in culture that have taxol-like activity.

Taxol activity is also confirmed in cell lines by showing that extracts have the ability to stabilize microtubule activity in the same manner as taxol. Another means of identification of taxol is accomplished by extracting roots, purifying this extract by HPLC and identifying taxol by utilizing H^1 and 2-D high resolution nuclear magnetic resonance (NMR) analysis (Falzone *et al.*, 1992).

Different hydroponic systems and bioreactor designs are available to maximize growth rates, taxol, active derivatives of taxol, cephalomannine, baccatin and other precursors of taxol made by each of the root cultures and secreted into solution (Bloom, 1989; Hilton *et al.*, 1988). By optimizing environmental conditions such as light levels, gas compositions/dissolved oxygen and/or CO_2, temperature, plant hormones, pH of the growth media,

nutrients, vitamins and removal of taxol from the media, desired compound extraction may be maximized.

The levels of taxol found in the roots of the present invention are at least 2- to 4-fold higher than in other parts of the plant (Table 1). These levels may be increased further. In general for a variety of root cultures, it has been shown that cultures producing low or undetectable levels of secondary products are elevated by a variety of means including optimization of culture conditions, precursor feeding, use of biotic (Heinstein, 1985; Fukui *et al.*, 1990), and abiotic elicitors including the use of ultra-violet irradiation (Kartusch & Mittendorfer, 1990) and heavy metal ions (Threlfall & Whitehead, 1988). The addition of precursors to roots have also been shown to increase secondary metabolite production. Precursor feeding, biotic and abiotic elicitors as defined herein, are suitable for use with *Taxus* root culture.

The induction of mutations in roots by irradiation and chemical mutagens may result in roots which overproduce taxol, 10-deacetyl baccatin III, 7-epi-10-deacetyl baccatin III, baccatin III, 7-epi baccatin III, 9-dihydro-13-acetyl baccatin III, cephalomannine, 10-deacetyl taxol, 7-epi10-deacetyl taxol, 7-epi taxol, other known and unknown natural derivatives of these compounds, and other related taxanes including precursors and intermediates in the biochemical pathway resulting in taxol production, and active derivatives of taxol. In addition, biotic and abiotic elicitors, feeding of precursors, and other elicitation techniques in shake cultures and large scale bioreactors coupled with large scale purification may increase product yield.

The present invention will be illustrated in further detail in the following examples. These examples are included for illustrative purposes and should not be considered to limit the present invention.

EXAMPLE 1

Preparation of a Callus Cell Line G1 from *Taxus*

Newly appearing, succulent (juicy) stem growth from *Taxus media cv.* Hicksii was harvested from trees located on The Pennsylvania State University, University Park Campus in the spring and immediately processed. The needles were removed from the stems and the stems cut into 3 cm sections. The young succulent stem tissue was selected because it contains the highest levels of taxol in all species of *Taxus* tested. The explants were surface sterilized for 20 seconds in ethanol and immediately placed in 10% bleach solution (v/v) plus Tween 20 for 20 minutes, rinsed 3X with sterile water and sectioned under aseptic conditions. Other standard surface sterilization procedures are also suitable. The surface sterilized stems were cut into sections under aseptic conditions and placed on Gamborg's B5 media plus and minus 2,4-D, kinetin or 2,4-D plus kinetin in the light and dark at 25°C. Undifferentiated callus tissue was initially slow growing, however, through many subcultures a friable yellowish colored cell line designated G1 with stable growth characteristics was obtained. This cell line grows on Gamborg's B5 media (Table II) in the dark at 25°C without the supply of any exogenous hormones.

The cell line G1 was maintained on media supplemented with casein hydrolysate, glucose and fructose, which were found to significantly increase the growth rate of this cell line (Table III).

Other cell lines were derived from G1. The callus cell line G1 was subcloned on modified Gamborg's B5 media shown in Table III. The callus cell line was then transferred to membrane rafts containing liquid media shown in Table II at 25°C in the dark. This cell line was subcloned and a cell line with stable growth characteristics was derived (CR1) (See Example 2).

EXAMPLE 2

Characteristics of a Taxol-Producing Cell Line CR-1 Prepared from Callus According to the Methods of the Present Invention

A callus-derived cell line designated CR-1, derived from a callus designated G1, has the following characteristics: grows in cultures wherein the nutrient medium is Gamborg's B5 medium; grows in the dark at 20-25°C; does not require exogenous plant hormones (is a habituated line); shows increased growth rate compared to its baseline by addition to medium of casein hydrolysate, glucose and fructose to the medium (see Table III); exhibits a doubling time of from 5-16 days; and shows stable growth in culture. The cell line was capable of secreting taxol, active derivatives of taxol, cephalomannine, baccatin and other precursors of taxol into the growth medium.

The callus-derived cell line designated CR1 produced rapid growth of callus after subculturing: three grams of callus seeded on a membrane raft produced 50-70 grams of callus within 7-10 weeks, with no subculturing, just the addition of fresh medium.

Authentic taxol and cephalomannine have retention times of 31 and 25 minutes, respectively. Callus samples such as CR-1 have retention times corresponding to authentic taxol and cephalomannine, however, suspension lines such as S2 have peaks which are shifted, eluting at 32 and 25 minutes respectively. This shift in retention times led to difficulty in purification of these compounds from suspension lines. This is not a problem with root cultures.

EXAMPLE 3

Characteristics of Taxol-Producing Suspension Cell Lines S3 and SR-1 Prepared According to the Methods of the Present Invention

Two suspension culture lines were also produced by the methods of the present invention from the callus cell line G1. A suspension culture line S3 was derived with stable growth characteristics when grown at 25°C in the

dark with modified Gamborg's B5 media shown in Table IV. Suspension cell lines produced in this manner were typically slow growing, however, through subcloning the cell line S3 was obtained and had a moderate growth rate. This cell line (S3) produces taxol, its precursors and active derivatives of taxol and secretes these chemicals into the solution and can easily be removed from the solution using exchange resins.

Another suspension culture cell line designated SR-1 was obtained in a unique way from CR1 callus lines grown on membrane rafts. A fine cell suspension was found in the nutrient solution in which the callus CR1 was growing on membrane rafts. The suspension culture of CR1 was produced using methods of the present invention wherein cells sloughed off callus cell line CR1 into suspension in the nutrient medium, were grown in 1 L shaker flasks with agitation in the dark at about 24-26°C in Gamborg's B5 medium as shown in Table IV. The suspension culture was designated SR-1. Suspension culture SR-1 was capable of secreting taxol, active derivatives of taxol, cephalomannine, baccatin and other precursors of taxol into the growth medium.

EXAMPLE 4

Characteristics of a Taxol-Producing Cell Line LG3 Prepared According to the Methods of the Present Invention

A sample of cells from cell line G1 (EXAMPLE 1) which grows in the dark was transferred to a light cycle of 12 hours light, 12 hours dark, at 25°C. Transfer to the light initially caused a slow down in growth rate and a brown discoloration. To avoid cell death this callus line was placed on several hormone regimes containing 2,4-D and kinetin. These cell lines were subcloned and a callus cell line designated LG3 which grows on Gamborg's B5 media was selected (Table III) in the light. LG3 requires exogenous plant hormones (1 ppm 2,4-D and 0.2 ppm kinetin) in order to grow. This callus line has been acclimated to membrane rafts and suspension cultures have been initiated. LG3 was subcultured on different

hormone regimes consisting of various combinations of 2-isopentyladenine and indole-3-butyric acid (0.1, 1.0 and 5.0 ppm). The cell line is capable of secreting taxol, active derivatives of taxol, cephalomannine, baccatin and other precursors of taxol into the growth medium.

The callus cell line LG3 was subcultured on 10 different hormone regimes of cytokinins and auxins in the light at 25°C. By using this method shoot formation was induced. This technique will be invaluable in regeneration of plants with superior taxol producing capabilities and as a means of elevating taxol levels in plants grown under aseptic conditions. LGS-3 is a suspension culture derived from LG3.

EXAMPLE 5

Extraction and Analysis of Taxol and Taxol-related Compounds from *Taxus* Cultures

Taxol, cephalomannine and baccatin were identified in each of the *Taxus media cv.* Hicksii cell lines CR1, S3, SR1, and LG3 using HPLC analysis (see Materials and Methods). This was accomplished by making comparisons of retention times between plant samples and authentic standards. Taxol activity was also confirmed by showing that extracts have the ability to stabilize microtubules in the same manner as authentic taxol. The third line of evidence that taxol was contained in the cell lines was by purifying taxol on HPLC and identifying taxol this purified sample by nuclear magnetic resonance analysis. Derivatives of taxol and additional precursors were also identified by HPLC in each of cell lines. This was accomplished by extracting each of the cell lines, running the extracts on HPLC, collecting major peaks and testing these samples in *in vitro* microtubule stabilizing assays (see Materials and Methods).

The levels of taxol produced in unelicited cultures of *Taxus media cv.* Hicksii cell lines CR1, S3, SR1, and LG3 Taxol levels were 2-3 times higher than the levels found in extracts from young stems as determined by HPLC analysis. Even more interesting were the HPLC profiles

revealing previously unidentified peaks with taxol-like activity, and profile differences among cell lines. For example, differences in the HPLC chromatogram profiles of cell lines SR-1 and S-3 were apparent when compared with the HPLC profile of cell line CR-1 under similar conditions. Peak 4, a peak which exhibits taxol-like activity and which is present in CR-1, is diminished or absent in S-3. Peak 5 is present in CR-1 but diminished in SR-1. Interestingly, neither peaks 4 nor 5 found in CR-1 was detected in direct extracts of *T. media* Hicksii, under the conditions used to detect the peaks of callus and culture cell extracts.

It is expected that other profiles will result if different growth parameters of cultures are used or different HPLC methods are applied. The methods for CR-1 are described herein. Other methods based on hydrophobicity or other distinguishing methods for ring-containing structures are suitable.

EXAMPLE 6

Elicitation and Precursor Feeding to Increase Yield of Taxol and Taxol-Related Compounds from Cultures

The suspension culture lines S3 and SR1 are grown in 1L flasks in Gamborg's B5 media (Table IV). Biotic (autoclaved fungal cell wall fragments and spores) and abiotic (salts of heavy metals: vanadate, lead and mercury) elicitation are suitable as elicitors. Both cell lines and elicitation methods may result in elevated levels of taxol, its precursors and taxol derivatives.

Roots grown both *in vivo* and *in vitro* were elicited with biotic (autoclaved fungal cell wall fragments and spores) and abiotic (salts of heavy metals: vanadate, lead and mercury) stresses both of which are suitable as elicitors. All *Taxus* species/cultivars evaluated were elicited to different degrees and different taxanes were increased.

Roots which were fed precursors and/or intermediates in the taxol biochemical pathway (i.e. acetate, mevalonic acid) resulted in an increase in taxol and a wide range

of taxanes, the specific levels depending upon the *Taxus* species/cultivar used.

EXAMPLE 7

Induction of Mutations in *Taxus* Cultures to Increase Taxol and Related Taxane Yield

In order to induce mutations which produce high levels of taxol, its precursors and active derivatives of taxol in cell lines CR1, S3, SR1 and LG3 are subjected to UV irradiation treatment and chemical mutagens such as EMS. Selection criteria and methods of culturing taxol-producing cell lines are according to those disclosed herein. In this fashion, mutations that increase the output of the taxol-producing cells, may be selected.

In order to induce mutations in *Taxus* roots which produce high levels of taxol, its precursors and active derivatives of taxol they are subjected to UV irradiation treatment and chemical mutagens such as EMS. In addition to induced mutations spontaneous mutations which occur naturally are also selected for. Selection criteria and methods of culturing taxol and related taxane producing roots are according to those disclosed herein. In this fashion, mutations that increase the output of the taxol and related taxane-producing roots, may be selected.

EXAMPLE 8

Cell Line and Root Storage in Inactive States

A novel method of storage preferred for *Taxus* cultures was to reduce incubation temperature to 4-10°C. Growth was slowed to a hibernation-like state. To return cells or roots to normal, the temperature was slowly raised to the optimum for growth.

The callus cell lines CR1 and LG3 are maintained on membrane rafts placed inside Magenta GA-7 vessels, while shaker cultures of S3 and SR1 are maintained in 1L shaker culture flasks and subcultured every 2 weeks. Cryopreservation for long term storage of cell cultures CR1, LG3, S3 and SR1 during the log phase of growth was accomplished by using standard cryopreservation methods using sorbitol and DMSO as the cryoprotectant.

Cryopreservation was shown to have great potential for long term preservation of cell cultures whose biosynthetic capacity for synthesis and accumulation of secondary metabolites remain intact following removal from storage.

The root cultures are maintained on membrane rafts placed inside Magenta GA-7 vessels and in shaker cultures are maintained in 1L shaker culture flasks and subcultured every 2 weeks. Cryopreservation for long term storage of root cultures during the log phase of growth was accomplished by using standard cryopreservation methods using sorbitol and DMSO as the cryoprotectant. Cryopreservation was shown to have great potential for long term preservation of roots whose biosynthetic capacity for synthesis and accumulation of secondary metabolites remain intact following removal from storage.

EXAMPLE 9

In Vitro Tissue Culture Derived from *Taxus* Roots

After stable culture growth is achieved, the root cultures are kept on maintenance medium. For some of the root cultures, habituation occurs, that is, the cultures are maintained without the need for hormonal supplements. the optimum culture conditions for the selected subclones are then determined by a multivariate analysis of factors which are likely to effect culture growth: presence or absence of light, gas composition, temperature, pH, medium supplements including plant hormones and sugars.

Aseptic normal and "hairy roots" are grown under the following conditions: on maintenance medium containing hormones for normal roots, and without hormones for "hairy roots," 25°C, pH 5.6, 21% O_2 on the remainder air, and kept in darkness, are used as a source of root tips for multivariate analysis of growth/taxane production. Several root tips are transferred to Gamborgs B5, Murashige and Skoog, McCown's Woody plant liquid media, and aerated.

The growth rates/taxane production are evaluated over a one month period of time, although other times may

be suitable depending on results. After the optimal basic nutrient composition has been determined, the effects of various levels of vitamins are evaluated. For example, if Gamborgs B5 media is found to be optimal, vitamins would be varied 1/2X, 1X, 2X, 3X and 4X of what is found in B5 media. After the vitamins are optimized, varying sugars such as sucrose, glucose or fructose are varied 1/2X, 1X, 2X and 3x. After the sugar concentration and type are optimized, the effects of temperatures ranging from 15 to 30°C are evaluated. After optimal temperature has been established, the effects of pH varying from 3.5 to 7.0 is evaluated.

After all of the previously mentioned parameters have been optimized, one half of the roots are kept in darkness, and the other half transferred to the light (50 μe m^{-2} s^{-1}) and allowed to acclimate. During the acclimation period, sugars are gradually removed from the growth media until the roots are capable of using photosynthesis as a carbon source at which time all sugars are removed.

After the roots are acclimated to the light, they are subjected to varying light levels from 50 to 100 mE μ^{-2} s^{-1} and growth rates/ taxanes evaluated. After light levels are optimized, they are kept constant while varying CO_2 levels from 0.03 to 3.0%. Carbon dioxide levels are also varied in dark grown roots, and growth rates/taxane levels are evaluated.

EXAMPLE 10

Extraction and Analysis of Taxol and Taxol-related Compounds from *Taxus* Roots

Taxol, 10-deacetyl baccatin III, 7-epi-10-deacetyl baccatin III, baccatin III, 7-epi baccatin III, 9-dihydro-13-acetyl baccatin III, cephalomannine, 10-deacetyl taxol, 7-epi10-deacetyl taxol, 7-epi taxol, other known and unknown natural derivatives of these compounds have been identified in each of the *Taxus* species/cultivars evaluated using HPLC analysis (see Materials and Methods). The quantity of taxol and

related taxane and the specific taxane differed with *Taxus* species/cultivar, for example 9-dihydro-13-acetyl baccatin III was found mainly in *Taxus canadensis*. This was accomplished by making comparisons of retention times between plant samples and authentic standards (FIGURE 1). Taxol activity was also confirmed by showing that extracts have the ability to stabilize microtubules in the same manner as authentic taxol. The third line of evidence that taxol was contained in the roots of *Taxus* plants was by purifying taxol on HPLC and identifying taxol this purified sample by nuclear magnetic resonance analysis. Derivatives of taxol and additional precursors were also identified by HPLC in roots from each of the different *Taxus* species/cultivars. This was accomplished by extracting each of the root cultures, running the extracts on HPLC, collecting major peaks and testing these samples in *in vitro* microtubule stabilizing assays (see Materials and Methods). There were also numerous taxanes which did not correspond to any of the standards used.

The levels of taxol produced in unelicited cultures of *Taxus* roots generally is 2-4 times higher than the levels found in extracts from other plant parts as determined by HPLC analysis. Even more interesting were the HPLC profiles from root extracts revealing previously unidentified peaks with taxol-like activity, and profile differences among roots obtained from different *Taxus* species/cultivars. For example, differences in the HPLC chromatogram profiles of *Taxus media, Taxus canadensis* and *Taxus cuspidata* were apparent when compared as shown in FIGURES 3-5. Other profiles not included show a wide range of differences between species/cultivars.

EXAMPLE 11

Use of Hydroponics for the Growth of *Taxus* Roots to Produce Taxol and Related Taxanes

The growth of roots in hydroponics by growing plants in aerated nutrient solution, nutrient film technique and other hydroponic systems results in taxol and related taxane production. Alternatively, new systems may be

designed and evaluated. The proper adjustment of environmental conditions such as gas composition, dissolved oxygen, rate of sparging and speed of agitation, temperature, light, pH of the growth medium, nutrients and organic supplements, removal of secondary products from the medium through the use of resins and elicitation are required to optimize the systems.

The growth of the roots are suitable in a "nutrient film system." This system has nutrient solution flowing over the membrane which supports the roots and is recirculated. Nutrients can be added as needed by measuring conductivity, and the presence of taxol, taxol derivatives and precursors in the medium can be assayed by sampling from the solution in line. This scale-up system is analogous to the growth of the roots on membrane rafts. This system can also be optimized by having the nutrient medium sprayed onto the roots intermittently through a system consisting of a nozzle, a peristaltic pump and a timer, thereby supplying the nutrients more uniformly to the entire root mass and also by increasing nutrient uptake, efficiency, and minimizing nutrient use.

Rooted cuttings are generally transferred to one quarter strength Hoagland solution where they are allowed to acclimate for a period of one month under the following conditions: 21% oxygen/0.03% carbon dioxide/remainder nitrogen bubbled through the nutrient solution, 20 to 25°C, pH 4 to 5 and no plant growth regulator treatments. The top portion of the plant is maintained at 60 to 90% relative humidity, 50 $\mu E\ m^{-2}\ s^{-1}$ irradiance, ambient levels of CO_2/O_2 and 20 to 25°C. The growth rates are monitored during this acclimation period, generally about one month, by the procedure outlined by Arteca et al. (1985).

After the acclimation period, the total nutrients are varied by evaluating 1/4, 1/2, 3/4, and full strength Hoagland solution while keeping constant the other

parameters previously mentioned. Growth rates/taxane production are then evaluated.

After the basic nutrient composition has been determined, it is incorporated into the one month acclimation period, and another set of parameters are evaluated. The next set of parameters evaluated following acclimation of plants are the effects of varied levels of nitrogen, potassium or phosphorous. For example if 1/2 strength Hoagland is found to be optimal, nitrogen, potassium or phosphorous levels are varied 1/2, 1X, 2X, and 4X the levels found in 1/2 Hoagland solution, and the growth rates/taxanes are evaluated.

After nutrients are optimized, varying levels of CO_2 from 0.03% to 5% are bubbled through the nutrient solution and the growth rate and taxane production are evaluated. After CO_2 levels are optimized, all of the previously mentioned parameters which have been optimized are kept constant and the temperature is varied from 15 to 25°C, after which growth rates and taxane production are evaluated.

After all parameters are optimized in the root zone, these are kept constant during the acclimation period. The top portion of the plant is subjected to light levels from 50 to 500 $\mu E\ m^{-2}\ s^{-1}$, and growth rates and taxane production are evaluated. After light levels are optimized, they are kept constant while varying CO_2 levels from 0.03 to 0.3%, and evaluating growth rates and taxane production. After light and CO_2 levels have been optimized, they are kept constant, and hormone treatments are made to increase biomass. This can be accomplished in two ways, either via the roots or as foliar sprays. The range of concentrations applied differs between root and shoot applications, with the root concentrations always being lower.

In order to maximize biomass production of both roots and shoots of hydroponically grown plants, gibberellins, cytokinins or brassinosteroids are applied to the roots at a concentration from 1 to 100 ppm for

gibberellins, and 0.01 to 1 ppm for cytokinins or brassinosteroids. When using foliar applications gibberellins are applied over 100 to 500 ppm range, and brassinosteroids or cytokinins are applied over a 1 to 10 ppm range. Following treatment, growth rates and taxane production are evaluated.

After sufficient biomass is obtained, growth of the top portion of the plant is slowed by applying growth retardants such as Cycocel to the foliage at concentrations ranging from 500 to 1000 ppm, and then evaluating growth rates and taxane production.

EXAMPLE 12

Use of Cultures or Roots in Bioreactors to Produce Taxol and Taxol-Related Compounds

The growth of suspension cell line SR1 in airlift, stirred and perfusion bioreactors results in taxol production. Alternatively, new reactors may be designed. The proper adjustment of environmental conditions such as gas composition, dissolved oxygen, rate of sparging and speed of agitation, temperature, pH of the growth medium, nutrients and organic supplements, removal of secondary products from the medium through the use of resins and elicitation are required to optimize the systems.

The growth of the callus line CR1 is suitable in a "membrane flow" bioreactor. This system has nutrient solution flowing over the membrane which supports the callus and is recirculated. Nutrients can be added as needed by measuring conductivity and the presence of taxol, taxol derivatives and precursors in the medium can be assayed by sampling from the solution in line. This scale-up system is analogous to the growth of the callus cell line CR1 on membrane rafts. This system can also be optimized by having the nutrient medium sprayed onto the callus intermittently through a system consisting of a nozzle, a peristaltic pump and a timer, thereby supplying the nutrients more uniformly to the entire callus mass and also by increasing nutrient uptake, efficiency, and minimizing nutrient use.

The growth of roots in airlift, stirred and perfusion bioreactors results in taxol and related taxane production. Alternatively, new reactors may be designed. The proper adjustment of environmental conditions such as gas composition, dissolved oxygen, rate of sparging and speed of agitation, temperature, light, pH of the growth medium, nutrients and organic supplements, removal of secondary products from the medium through the use of resins and elicitation are required to optimize the systems.

The growth of the roots are suitable in a "membrane flow" bioreactor. This system has nutrient solution flowing over the membrane which supports the roots and is recirculated. Nutrients can be added as needed by measuring conductivity and the presence of taxol, taxol derivatives and precursors in the medium can be assayed by sampling from the solution in line. This scale-up system is analogous to the growth of the roots on membrane rafts. This system can also be optimized by having the nutrient medium sprayed onto the roots intermittently through a system consisting of a nozzle, a peristaltic pump and a timer, thereby supplying the nutrients more uniformly to the entire root mass and also by increasing nutrient uptake, efficiency, and minimizing nutrient use.

While the invention is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings and herein are described in detail. It should be understood, however, that it is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

MATERIALS AND METHODS

1. Microtubule stabilizing bioassay

The microtubule stabilizing bioassay is used to screen for the ability of a composition to stabilize the

formation of microtubules. There is a direct, predictive correlation between results in the microtubule bioassay and the chemotherapeutic action of a composition in cancer model assays. Therefore, the microtubule stabilizing bioassay is recognized by those of skill in the art as a screening test for effective chemotherapeutic agents for further clinical trials.

To conduct the bioassay, neuronal microtubule (MT) proteins are prepared according to the methods of Shelanski et al. (1973) using fresh calf brains. Endogenous microtubule-associated proteins (MAPs) are removed from MT proteins which have been cycled one time, by cycling 4 times in the presence of 1 M glutamate (Hamel and Lin, 1981). After completion of the cycling, the last tubulin pellet obtained is solubilized in cold PM buffer and is further purified by phosphocellulose chromatography according to Weingarten *et al.* (1975).

An assay using dark-field microscopy is performed as follows:

(1) the test extracts are those solubilized in PM buffer as described above.

PM Buffer: 50 mM 1,4-piperzinediethanesulfonic acid (Pipes) at pH 6.9; 1 mM magnesium sulfate; 1 mM ethylene glycol-bis (b-aminoethyl ether)-N,N,N',N'-tetraacetic acid [EGTA] tubulin and a Mg-GTP stock.

2. Physical Selection Criteria for Callus and Subclones to Produce a Taxol-producing Cell Line (see FIGURES 2 and 3)

Approximately 90% of the *Taxus* stem explants showed callus induction after about 2-4 weeks of initiation. Yellow colored callus were selected, separated from the explant, and subcultured. Callus characteristically show initial slow growth, only doubling in about 3-6 months or longer. Another difficulty to be overcome is that most callus turn a brownish color within 1-3 weeks after subculture, and eventually die. Red-colored exudates were another sign of impending cell death.

Clumps of yellow-colored, friable callus were selected for subculturing. Red and brown colored clumps

were not selected. Selectable clumps were often surrounded with brown-colored (dead) callus cells,the live cells sometimes appearing as nodules protruding from a brown-colored mass of callus.

3. Extraction of Taxol and Related Taxanes

Roots / root cultures were harvested, freeze-dried, and extracted with 1:1 mix of methanol and methylene chloride by homogenizing in an Omni-mix homogenizer (Omni International, Waterbury, Connecticut) followed by sonication for 15 minutes. The extract was filtered through a Whatmann # 1 filter paper and the filtrate was concentrated to dryness *in vacuo*. The residue was then partitioned between methylene chloride and water and the methylene chloride fraction was concentrated to dryness under a stream of nitrogen. The final pellet/residue was then resuspended in acidic methanol (0.1% acetic acid), and filtered through a 0.2 micron nylon filter for high performance liquid chromatography (HPLC) analysis. The use of solid phase extraction cartridges (C_{18} Sep-pak cartridges; Waters, Milford, Massachusetts) was unnecessary since the root extracts are much cleaner compared to needles, stems, bark, callus and cell suspension cultures (Wickremesinhe and Arteca, 1993c)

4. Identification by HPLC

Analytical HPLC was performed on a Curosil G 4 m column (4.6 mm x 250 mm; Phenomenex, Torrance, CA) with a phenyl guard module (Rainin Instrument Co. Inc., Woburn, Massachusetts). A mobile phase consisting of 10 mM acetate buffer (pH = 4.0) : acetonitrile (56:44), at a flow rate of 0.6 ml per minute was used. Taxol was detected by monitoring absorbance at 227 nm.

Duplicate injections were made from every sample and the average of the two peak areas was used to quantify taxol and cephalomannine. Authentic samples of 10-deacetyl baccatin III, 7-epi-10-deacetyl baccatin III, baccatin III, 7-epi baccatin III, 9-dihydro-13-acetyl baccatin III, cephalomannine, 10-deacetyl taxol, 7-epi10-deacetyl taxol, 7-epi taxol, and taxol, were used for the identification of peaks. A linearity curve was

established for all the major taxanes by injecting amounts of authentic standards ranging from 0.001 to 10 mg per injection. Peaks for taxol were confirmed by collecting them and running then in the microtubule stabilizing bioassay and NMR analysis.

5. Nuclear Magnetic Resonance Spectroscopy (NMR)

Authentic taxol and taxol purified from roots were solubilized in deuterated methylene chloride and the 1H and 13C NMR spectra were analyzed by one- and two dimensional methods described by Falzone *et al.* (1992).

6.

TABLE II Composition of Gamborgs B5 medium.

	mg/L
Basal Salts	
Ammonium sulfate	134.000
Boric acid	3.000
Calcium chloride anhydrous	113.240
Cobalt chloride hexahydrate	0.025
Cupric sulfate pentahydrate	0.025
Disodium EDTA dihydrate	37.250
Ferrous sulfate heptahydrate	27.850
Magnesium sulfate anhydrous	122.090
Manganese sulfate monohydrate	10.000
Potassium iodide	0.750
Potassium nitrate	2500.000
Sodium molybdate dihydrate	0.250
Sodium phosphate monobasic anhydrous	130.500
Zinc sulfate heptahydrate	2.000
Vitamins	
Myo-inositol	100.0
Thiamine HCl	10.0
Pyridoxine	1.0
Nicotinic acid	1.0
Sugars	
Sucrose	20,000.0

7.

TABLE III Modified Gamborgs B5 medium for growth of callus.

Basal Salts	
	mg/L
Ammonium sulfate	134.000
Boric acid	3.000
Calcium chloride anhydrous	113.240
Cobalt chloride hexahydrate	0.025
Cupric sulfate pentahydrate	0.025
Disodium EDTA dihydrate	37.250
Ferrous sulfate heptahydrate	27.850
Magnesium sulfate anhydrous	122.090
Manganese sulfate monohydrate	10.000
Potassium iodide	0.750
Potassium nitrate	2500.000

Sodium molybdate dihydrate	0.250
Sodium phosphate monobasic anhydrous	130.500
Zinc sulfate heptahydrate	2.000
Vitamins	
Myo-inositol	100.0
Thiamine HCl	10.0
Pyridoxine	1.0
Nicotinic acid	1.0
Sugars	
Sucrose	20,000.0
Glucose	2,500.0
Fructose	2,500.0
Casein hydrolysate	1,000.0

8.

TABLE IV Modified Gamborgs B5 medium for growth of cell suspensions.

Basal Salts	**mg/L**
Ammonium sulfate	134.000
Boric acid	3.000
Cobalt chloride hexahydrate	0.025
Cupric sulfate pentahydrate	0.025
Disodium EDTA dihydrate	37.250
Ferrous sulfate heptahydrate	27.850
Magnesium sulfate anhydrous	122.090
Manganese sulfate monohydrate	10.000
Potassium iodide	0.750
Potassium nitrate	2500.000
Sodium molybdate dihydrate	0.250
Sodium phosphate monobasic anhydrous	130.500
Zinc sulfate heptahydrate	2.000
Vitamins	
Myo-inositol	200.0
Thiamine HCl	20.0
Pyridoxine	2.0
Nicotinic acid	2.0
Sugars	
Sucrose	20,000.0
Glucose	2,500.0
Fructose	2,500.0
Casein hydrolysate	200.0

9.

TABLE V

Cell line	Doubling time (days)	Taxol %
CR-1	10-15	0.001-0.030
SR-1	5-9	0.001-0.016
S-3	8-12	0.001-0.008
LG-3	15-19	0.001-0.020
LGS-3	13-16	0.001-0.012

References

The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

Adler, P.R., and Wilcox, G.E. 1987. Hydroponic tomato yield affected by chloromequat chloride , seedling time, and transplant maturity. J. Amer. Soc. Hort. Science 112, 198-201.

Arbuck S.G. (1992) Current status of the clinical development of taxol. Second National Cancer Institute Workshop on Taxol and *Taxus*: September 23-24, 1992, Alexandria, Virginia (abstract)

Bloom, A.J. Continuous and steady state nutrient absorption by intact plants, pp. 147-163. Applications of Continuous and Steady-State Methods to Root Biology, Edited by J. G. Torrey and L. J. Winship, Kluwer Academic Publishers 1989.

Blume, E. 1991. Government moves to increase taxol supplies (news). J. Nat. cancer. Inst. 83, 1054-1056.

Borman, S. 1991. Scientists mobilize to increase supply of anticancer drug taxol. Chemical & Engin. News 69, 11-18.

Brentlinger, D. 1992. Tomatoes in perlite. A simplified hydroponic system. Amer. Vegetable Grower 40, 50.

Brodelius, P. Immobilized plant cells as a source of biochemicals, In: Bioreactor immobilized enzymes and cells; Ed: M. Moo-Young. Elsevier Applied Science, London and New York, 1988, pp. 167-196.

Brodelius, P. The potential role of immobilization in plant cell biotechnology. Trends in Biotechnology, 3:280-285 (1985).

Chauviere, G., Guenard, D., Picot, F., Senihl, V., and Potier, P. 1981. Analyse structurale et etude biochimique de produits isoles de l'if *Taxus baccata* L. (taxacees). C.R. Acad. Sci. Paris, Ser II, 193, 501-503.

Christen *et al.*, Production of Taxol or Taxol-like Compounds in Cell Culture. U.S. Patent No. 5,019,504 (1991).

Denis, J-N., A.E. Green, D. Guenard, F. Gueritte-Voegelein, L. Mangatal and P. Potier. A highly efficient, practical approach to natural taxol. J. of Amer. Chem. Soc. 110:5917-5919 (1988).

Donehower, R.C., E.K. Rowinsky, L.B. Grochow, S.M. Longnecker and D.S. Ettinger. Phase I trial of taxol in patients with advanced cancer. Cancer Trt. Reports 71(12):1171-1177 (1987).

Einzig, A.I., Wiernik P.H., and Schwartz E.L. 1991. Taxol: a new agent active in melanoma and ovarian cancer. Cancer Treat. Res. 58, 89-100.

Ettinger, D.S. (1992) Taxol in the treatment of lung cancer. Second National Cancer Institute Workshop on Taxol and *Taxus*: September 23-24, 1992, Alexandria, Virginia (abstract)

Falzone, C.A., Benesi, A.B., and Lecomte, J.T.J. 1992. Characterization of taxol in methylene chloride by NMR spectroscopy. Tetrahedron Lett. 33, 1169-1172.

Fett-Neto A.G., DiCosmo, F., Reynolds, W.F. & Sakata, K. (1992). Cell culture of *Taxus* as a source of the antineoplastic drug taxol and related taxanes. Bio/Technol. 10:1572-1575.

Fukui, H., M. Tania and M. Tabatu. Induction of shikonin by endogenous polysaccharides in Lithospermum erythrorhizon cell suspension cultures. Plant Cell Reports 9:73-76 (1990).

Gamborg, O.L., R.A. Miller and K. Ojima. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50:151-158 (1968).

Gueritte-Voegelein, F., G. Daniel, and P. Potier. Taxol and derivatives: a biogenetic hypothesis. Journal of Natural Products, 50(1):9-18 (1987).

Gunby, P. (1992) National Cancer Institute making more taxol available for refractory ovarian and breast cancers. J. Amer. Med. Assoc. 268:1824-1825.

Hamel, E. and C.M. Lin. Glutamate-induced polymerization of tubulin: characteristics of the reaction and application to the large-scale purification of tubulin. Arch. Biochem. Biophys., 209:29-40 (1981).

Heinstein, P.F. Future approaches to the formation of secondary natural products in plant cell suspension cultures. J. Nat. Prod. 48:1-9 (1985).

Hicklenton, P.R., Blatt, C.R., and O'Regan, R.J. 1987. Hydroponic production of cut chrysanthemums: a commercial trial. HortScience 22, 287-289.

Hilton, M.G., Wilson, P.D.G., Robbins, R.J., and Rhodes, M.J.C. Transformed root cultures- Fermentation aspects, pp. 239-245. In Manipulating Secondary Metabolism in Culture/ Edited by Richard J. Robbins and Michael J. C. Rhodes, Cambridge University Press.

Hoagland, D.R. and Arnon, D.I. 1938. The water culture method for growing plants without soil. University of California Exp. Cir. 347.

Holmes, F.A., Walters, R.S., Theriault, R.L., Forman, A.D., Newton, L.K., Raber, M.N., Buzdar, A.U., Frye, D.K., and Hortabagyi, G.N. 1991. Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. J. Nat. Cancer. Inst. 83, 1797-1805.

Holmes, F.A., Waters, R., Valero, V., Theriault, R., Booser, D., Buzdar, A., Gibbs, H., Frye, D. & Hortobagyi, G. (1992) The M. D. Anderson experience with taxol in metastatic breast cancer. Second National Cancer Institute Workshop on Taxol and *Taxus*: September 23-24, 1992, Alexandria, Virginia (abstract)

Holton, R.A., Liu, J.H., Gentile, L.N. & Beidiger, R.J. (1992) Semi-synthesis of taxol. Second National Cancer Institute Workshop on Taxol and *Taxus*: September 23-24, 1992, Alexandria, Virginia (abstract)

Horowitz, S.B., L. Lothsteia, J.J. Manfredi, W. Mellado, J. Parness, S.N. Roy, P.B. Schiff, L. Sorbara and R. Zeheb. Taxol: Mechanism of action and resistance. Annals New York Academy of Sciences, New York: The academy, vol. 466, pp. 733-743 (1986).

Howard, W.D. and S.N. Timasheff. Linkages between the effects of taxol, colchicine and GTP on tubulin polymerization. J. of biological chemistry, 263(3):1342-1346 (1988).

Huang, C.H.O., D.G.I. Kingston, N.F. Magri and G. Samaranayake. New taxanes from *Taxus brevifolia*, 2. J. of Natural Products 49(4):665-669 (1986).

Ishimaru, K., Hirosi, M., Takahashi, K., Koyama, K., and Shimomura, K. 1992. Tannin production in root cultures of *Sanguisorba officinalis*. Phytochemistry 29, 3827-3830.

Ishimaru, K., Yoshimatsu, K., Yamakawa, T., Kamada, H., and Shimomura, K. 1992. Phenolic constituents in tissue cultures of *Phyllanthus niruri*. Phytochemistry 31, 2015-2018.

Jha, S., Sahu, N.P., Sen, J., Jha, T.B., and Mahato, S.B. 1991. Production of emetine and cephaeline from cell suspension and excised root cultures of *Cephaelis ipecacuanha*. Phytochemistry 30, 3999-4003.

Kanabus, J., Bressan, R.A., Carpita, N.C. 1986. Carbon assimilation in carrot cells in liquid culture. Plant Physiol. 82, 363-368.

Kartusch, R. and B. Mittendorfer. Ultraviolet radiation increases nicotine production in Nicotiana callus cultures. J. Plant Physiol, 36:110-114 (1990).

Kingston, D.G.I., D.R. Hawkins and L. Ovington. New taxanes from *Taxus brevifolia*. J. Natural Products, 45(4):466-470 (1982).

Knight, S.L. and Mitchell, C.A. 1987. Stimulating productivity of hydroponic lettuce in controlled environments with triacontanol. HortScience 22, 1307-1309.

Kratky, B.A., Bowen, J.E., and Hideo, I. 1988. Observations on a noncirculating hydroponic system for tomato production. HortScience 23, 906-907.

Lardizabal, R.D., and Thompson, P.G. 1988. Hydroponic culture, grafting, and growth regulators to increase flowering in sweet potato. HortScience, 23, 993-995.

Mangatal, L., Adeline, M.-T., Guenard, D., Gueritte-Voegelein, F., and Potier, P. 1989. Application of the vicinal oxyamination reaction with asymetric induction to the semisynthesis of taxol and analogues. Tetrahedron 45, 4177-4190

Markman, M. 1991. Taxol: an important new drug in the management of epithelial ovarian cancer. Yale J. Biol. Med. 64: 583-590.

McGuire, W.P. (1992) Taxol: past, present and future in epithelial ovarian cancer (EOC). Second National Cancer Institute Workshop on Taxol and *Taxus*: September 23-24, 1992, Alexandria, Virginia (abstract)

McGuire, W.P., E.K. Rowinsky, N.B. Rosenshein, F.C. Grumbine, D.S. Ettinger, D.K. Armstrong, R.C. Donehower. Taxol: A unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. Annals of Internal Medicine 111:273-279 (1989).

McLaughlin, J.L., R.W. Miller, R.G. Powell, and C.R. Smith Jr. 19-Hydroxybaccatin III, 10-deacetylcephalomannine, and 10-deacetyltaxol: New antitumor taxanes from *Taxus wallichiana*. J of Natural Products 44(3):312-319 (1981).

Miller, R.W., R.G. Powell, and C.R. Smith Jr. Antileukemic alkaloids from *Taxus wallichiana* Zucc. J. Organic Chemistry 46:1469-1474 (1981).

Miller, R.W., R.G., and Smith, C.R. Jr. 1981. Antileukemic alkaloids from *Taxus wallichiana* Zucc. J. Org. Chem. 46, 1469-1474.

Misawa, M., M. Hayashi and S. Takayama. Accumulation of antineoplastic agents by plant tissue cultures, In: Primary and Secondary Metabolism of Plant Cell Cultures; Ed. K.H. Neumann, W. Barz and E. Reinhard. Springer, Berlin Heidelberg 1985, vol 31, pp 235-246.

Mortley, D.G., Bonsai, C.K., and Loretan, P.A. 1991. Evaluation of sweet potato genotypes for adaptability to hydroponic systems. Crop Science 31, 845-847.

Murashige, T. and F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 15:473-497 (1962).

Ojima, I., Habus, I., Zhao, M., Georg, G.I., and Jayasinghe, L.R. 1991. Efficient and practical asymmetric synthesis fo the C-13 side chain, N-benzoyl-(2R,3S)-3-phenylisoserine, and its analogues via chiral 3-hydroxy-4-aryl-b-lactams through chiral ester enolateimine cyclocodensation. J. Org. Chem. 56, 1681-1683.

Parness, J. and S.B. Horowitz. Taxol binds to polymerized tubulin *in vitro*. L Cell Biol., 91(2, Pt. 1):479-487 (1981).

Payne, G.F., Shuler M.L. and P. Brodelius. (1987). Large Scale Plant Cell Culture. In: Large Scale Cell Culture Technology, Ed: Bjorn K. Lyderson. Hanser Publishers, Munich, Vienna, New York. pp. 194-229.

Persinos, G.J. 1991. NCI hopes nursery clippings will add kilos to its taxol stock. Washington Insight 4, 6.

Potrykus, I., Gene transfer to plants, Annu. Rev. *Plant Physiio* Plant Mol, Biol. 1991. 42: 205-225

Ringel, I., and Horowitz, S.B. 1991. Studies with RP 56976 (taxotere): A semisynthetic analogue of taxol. J. Nat. Cancer Inst. 83, 288-291.

Riou, J-F., Naudin, A., and Lavelle, F. 1992. Effects of taxotere on murine and human tumor cell lines. Biochem. and Biophys. Research Communications 187, 164-170.

Rowinsky, E.K. and Donehower, R.C. 1991. Taxol: Twenty tears later, the story unfolds. J. Nat. Cancer Inst. 83, 1778-1781.

Schiff, P.B. and S.B. Horowitz. Taxol stabilizes microtubules in mouse fibroblast cells. Proc. Nat. Acad. Sci. 77(3):1561-1565 (1980).

Sen, J., Sharma, A.K., Sahu, N.P., and Mahato, S.B. 1992. Production of forskolin in in vitro cultures of *Coleus forskohlii*. Planta Med. 58, 324-327.

Shelanski, M., F. Gaskin and C. Cantor. Microtubule assembly in the absence of added nucleotides. Proc. Nat. Acad. Sci. USA, 70:765-768 (1973).

Shimomura, K., Sauerwein, M., and Ishimaru, K. 1991. Tropane alkaloids in the adventitious and hairy root

cultures of solanaceous plants. Phytochemistry 30, 2275-2278.

Slichenmyer, W.J. and Von Hoff, D.D. 1991. Taxol: a new and effective anti-cancer drug. Anti-Cancer Drugs 2, 519-530.

Smidsrod, O. and G. Skjak-Braek. Alginate as immobilization matrix for cells. Trend in Bio/Technology, 8:71-78.

Snader, K. Overview of supply issues. Workshop on taxol and *Taxus*: Current and future perspectives. Sponsored by: Developmental Therapeutics Program, Division of cancer treatment, National cancer institute (1990). Abstract.

Smigel, K. 1992. Breast cancer and taxol top press and public interest: The cancer stories of 1991. J. Nat. Cancer. Inst. 84, 10-11.

Southton, I.W. and J. Buckingham. Dictionary of Alkaloids, Chapman & Hall, New York (1989), pp 602, 1024.

Strobel, G.A., Stierle, A., and van Kuijk, F.J.G.M. 1992. Factors influencing the in vitro production of radiolabelled taxol by Pacific yew, *Taxus brevifolia*. Plant Science 84, 65-74.

Stull, D.P. (1992) Current taxol production from yew bark and future production strategies. Second National Cancer Institute Workshop on Taxol and *Taxus*: September 23-24, 1992, Alexandria, Virginia (abstract)

Suffness, M. Discovery and early development of taxol. Workshop on taxol and *Taxus*: Current and future perspectives. Sponsored by: Developmental therapeutic program, Division of cancer treatment, National cancer institute (1990). Abstract.

Suffness, M. and G.A. Cordell. Antitumor alkaloids. The alkaloids, ed. A. Brossi. Academic Press, Inc. (1985), vol. XXV. pp 6-20 and 57-69.

Suffness, M. and Cordell, G.A. (1985). Antitumor alkaloids. In: Alkaloids vol. XXV. pp 6-18.
Suffness M. (1990). Discovery and early development of taxol. Workshop on Taxol and Taxum: Current and Future Perspectives. National Cancer Institute, June 1990. Abstract.

Sugimoto, Y., Sugimura, Y., and Yamada, Y. 1988. Effects of culture conditions on bisbenzylisoquinoline alkaloid production in cultured roots of *Stephania cepharantha*. Agric. Biol. Chem. 52,1495-1498.

Thigpen, T., J. Blessing, H. Bell *et al.*, Phase II trial of taxol as second-line therapy for ovarian carcinoma:

A gynecologic oncology group study: Proc. Am. Soc. Clin. Oncol. 9: 156 (1990).

Threlfall, D.R. and I.M. Whitehead. The use of metal ions to induce the formation of secondary products in plant tissue culture, In: Manipulating Secondary Metabolism in Culture, Ed; R.J. Robins and M.J.C. Rhodes. Cambridge University Press, Cambridge, 1988, pp.51-56.

Vidensek, N., P. Lim, A Campbell and C. Carlson. Taxol content in bark, wood, root, leaf, twig and seedling from several *Taxus* species. J. Nat. Prod. 53:1609-1610 (1990).

Wani, M.C., H.L. Taylor, M.E. Wall, P. Coggon and A.T. McPhail. Plant antitumor agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J. American Chemical society 93:2325-2327 (1971).

Weathers, P.J. and Giles, K.L. (1988). Regeneration of plants using nutrient mist culture. *In vitro* cellular & Developmental Biology. 24: 727-732.

Weingarten, M.D., A.H. Lockwood, S.-Y. Kwo and M.W. Kirschner. A protein factor essential for microtubule assembly. Proc. Nat. Acad. Sci. USA, 72:1858-1862 (1975).

Wender, P.A. (1992) Studies on the synthesis of taxol. Second National Cancer Institute Workshop on Taxol and *Taxus*: September 23-24, 1992, Alexandria, Virginia (abstract)

Wheeler, N.C., Jech, K., Masters, S., Brobst, S.B., Alvarado, A.B., Hoover, A.J. and Snader, K.M. 1992. Effects of genetic, epigenetic, and environmental factors on taxol content in *Taxus brevifolia* and related species. J. Nat. Prod. 55, 432-440.

Wickremesinhe, E.R.M. & Arteca, R.N. (1993c) Establishment of a habituated callus line from *Taxus media* cv. Hicksii : Optimization of growth and confirmation of taxol production. Submitted to Plant Cell Tissue Organ Culture.

Wickremesinhe, E.R.M. and Arteca, R.N. 1991b. Detection of taxol in immature *Taxus media* and *Taxus cuspidata* stems. HortScience 26, 717 (abstract).

Wickremesinhe, E.R.M. and Arteca, R.N. 1991a. Production of taxol in callus and cell suspension cultures of *Taxus media* 'Hicksii". In Vitro, 27, 109A (abstract).

Wickremesinhe, E.R.M & Arteca, R.N. (1993b) Callus induction from stem explants of *Taxus*. Submitted to Plant Cell Reports.

Wickremesinhe, E.R.M. and Arteca, R.N. 1993a. Establishment of fast-growing callus and root cultures of Cephalotaxus harringtonia. Plant Cell Rep. 12, 80-83.

Wickremesinhe, E.R.M. (1992) Callus and cell suspension cultures of *Taxus* as a source of taxol and related taxanes. PhD thesis. The Pennsylvania State University, University Park, Pennsylvania

Witherup, K.M., S.A. Look, M.W. Stasko, T.J. Ghiorzi and G.M. Muschik. *Taxus* spp. needles contain amounts of taxol comparable to the bark of *Taxus brevifolia*: analysis and isolation. J. Natural Products 53:1249-1255 (1990).

Zamir, L.O., Nedea, M.E., and Garneau, F.X. 1992. Biosynthetic building blocks of *Taxus canadensis* taxanes. Tetrahedron Lett. 33, 5235-5236.

Zamir, L.O., Nedea, M.E., Belair, S., Sauriol, F., Mamer, O., Jacqmain, E., Jean, F.I, and Garneau, F.X. 1992. Taxanes isolated from *Taxus canadensis*. Tetrahedron Lett. 33, 5173-5176.

What Is Claimed Is:

1. A method for producing a purified compound selected from the group consisting of taxol, a precursor or intermediate of the biochemical pathway resulting in taxol production, and a derivative of taxol, said method comprising:

(a) culturing a tissue explant from a plant of the genus *Taxus* under conditions which allow formation of a culture and production of the compound by the cultured cells;
and

(b) collecting the compound from the culture or from the conditioned medium of the culture.

2. The method of claim 1, wherein the tissue comprises a root or a young stem.

3. The method of claim 2, where the root is derived from a dormant plant.

4. The method of claim 1, further comprising enhancing production of secondary metabolites of the compounds by applying elicitation techniques during culturing.

5. The method of claim 3, wherein elicitation techniques comprise feeding taxol precursors to the culture.

6. The method of claim 5, wherein the taxol precursor is acetate.

7. The method of claim 4, wherein elicitation techniques comprise adding fungal extracts.

8. The method of claim 1, comprising further isolating and purifying of the collected compound to remove non-taxol-containing compounds and contaminants.

9. The method of claim 2, wherein the root is a hairy root.

10. The method of claim 9, wherein the hairy root is produced by infection of a non-hairy *Taxus* root with *Agrobacterium*.

11. The method of claim 1, wherein the culturing is by means of *in vitro* tissue culture of a root.

12. The method of claim 1, wherein the culturing is by means of hydroponic growth of roots.

13. The method of claim 1, wherein the compound is 9-dihydro-13-acetyl baccatin-3, and the plant of the genus *Taxus* is *Taxus canadensis*.

14. The method of claim 1, wherein *Taxus* is further defined as *Taxus media cv*. Hicksii.

15. The method of claim 1, wherein the compound is capable of inhibiting cell growth in a cancer model system or of producing positive results in a microtubule-stabilizing bioassay.

16. The method of claim 15, wherein the cancer model system comprises a cell line selected from a group consisting of B16 melanoma, MX-1 mammary xenograft, P388 leukemia, KB, and L1210 leukemia.

17. A method for establishing a cell line from a culture of *Taxus*, said method comprising the steps of:

(a) selecting a tissue source for an explant from the group consisting of a young stem and a root;

(b) culturing the explant in callus-inducing medium to form a callus;

(c) subculturing selected sections of the callus to promote subclone growth;

(d) selecting a subclone to develop a cell line; and

(e) culturing the cell line on a solid support in maintenance medium.

18. The method of claim 17, wherein *Taxus* is further defined as *Taxus media* cv. Hicksii.

19. The method of claim 17, wherein the callus-inducing medium comprises Gamborg's B5 medium supplemented with plant hormones.

20. The method of claim 19, wherein the plant hormones are selected from a group consisting of 2,4-D, kinetin and a combination of 2,4-D and kinetin.

21. The method of claim 17, wherein the selected sections of callus are selected based on criteria selected from the group consisting of high growth rate compared to growth rate of other callus, lack of red

coloring, said coloring indicating the presence of exudates which inhibit growth, presence of yellow coloring, and friability.

22. The method of claim 17, wherein the solid support for maintenance of the cell line comprises a membrane raft and the maintenance medium comprises modified Gamborg's B5 medium according to Table IV.

23. The method of claim 17, wherein the maintenance medium comprises casein hydrolysate, glucose and fructose.

24. The method of claim 23, wherein the concentration of glucose and fructose totals about 10-40 g/l.

25. The method of claim 17, wherein the maintenance medium comprises a sugar selected from a group consisting of sucrose, glucose and fructose, and wherein the concentration of said sugar is about 40-80 g/l.

26. The method of claim 23, wherein the concentration of casein hydrolysate is about 0.2-1.0 g/l.

27. The method of claim 17, wherein the root is derived from a plant in the dormant phase.

28. A purified compound that is the product of a process according to claim 1, wherein said compound is selected from the group consisting of taxol, a precursor or intermediate of the biochemical pathway resulting in taxol production, and a derivative of taxol.

29. A cell line, derived from a *Taxus* tissue, said cell line having the following characteristics:

(a) stable, long term growth in culture; and

(b) capability of expressing a compound selected from the group consisting of taxol, a precursor or intermediate of the biochemical pathway resulting in the production of taxol, and a derivative of taxol.

30. The cell line of claim 29, wherein *Taxus* comprises *Taxus media* cv. Hicksii.

31. The cell line of claim 29, wherein the compound is capable of inhibiting cell growth in cancer model systems or of producing positive results in a microtubule-stabilizing bioassay.

32. The cell line of claim 31, wherein the cancer model system comprises a cell line selected from a group consisting of B16 melanoma, MX-1 mammary xenograft, P388 leukemia, KB, and L1210 leukemia.

33. The cell line of claim 29, further defined as producing an amount of taxol that is at least about 1-2 fold increased above that produced by direct extraction from stems, leaves or bark of *Taxus media* cv. Hicksii.

34. A method of producing taxol, said method comprising:

(a) placing a cell line or a hydroponic root culture derived from *Taxus* in a bioreactor;

(b) operating the bioreactor so that taxol is produced by the cell line or root and is secreted into the nutrient medium of the bioreactor; and

(c) collecting the taxol from the medium.

35. The method of claim 34, wherein the cell line is produced according to claim 17.

36. A compound exhibiting microtubule-stabilizing activity, wherein said compound is selected from the group consisting of peaks eluting on an HPLC profile from a Curosil G column, number 40, of an extract from a stable *Taxus* cell line or a hydroponically grown root.

37. The compound of claim 36, wherein the *Taxus* cell line is CR-1 and the peak selected elutes at 31 minutes.

38. The compound of claim 36, selected from a group consisting of peaks A-K according to FIGURES 3-5.

FIG. 1

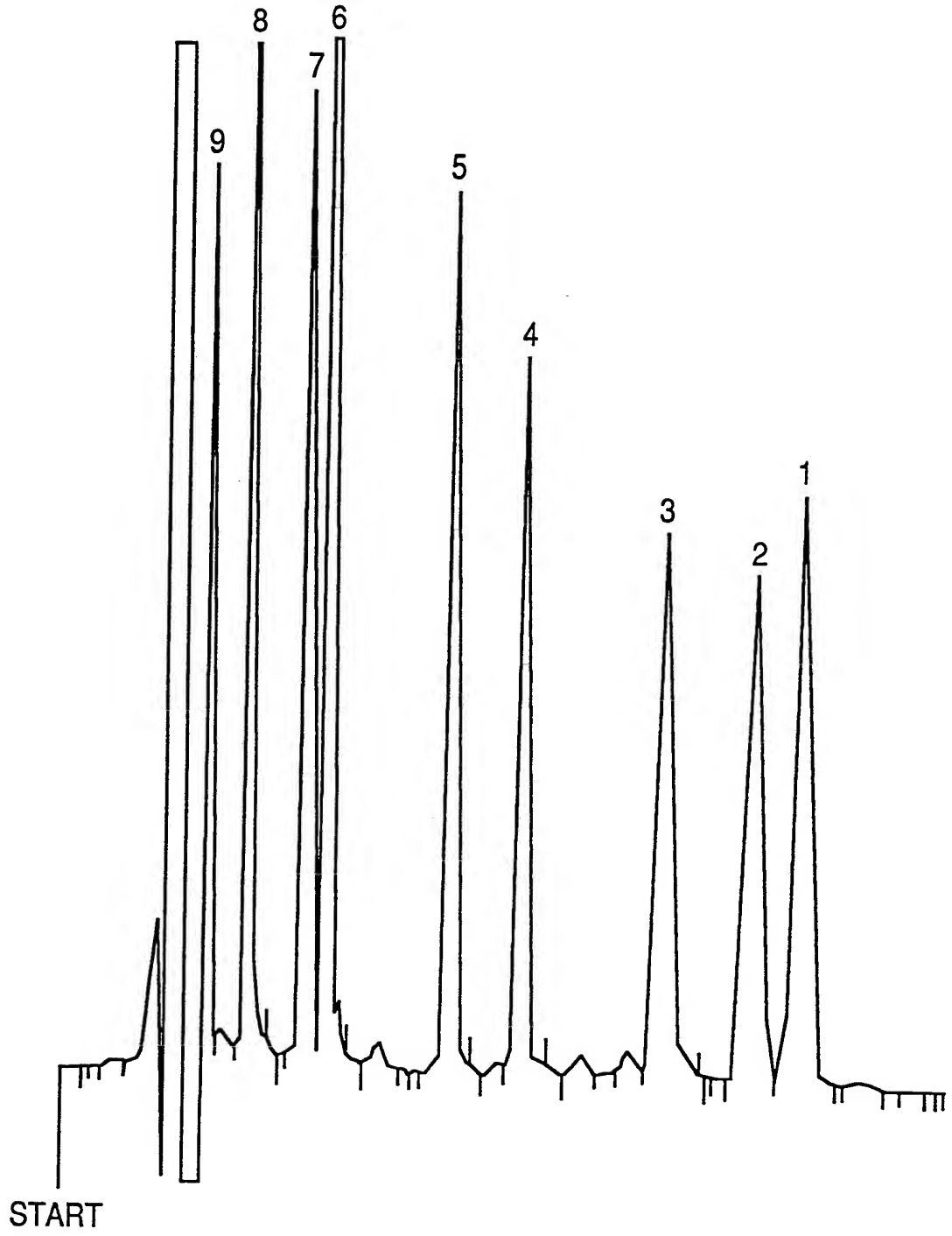


FIG. 2

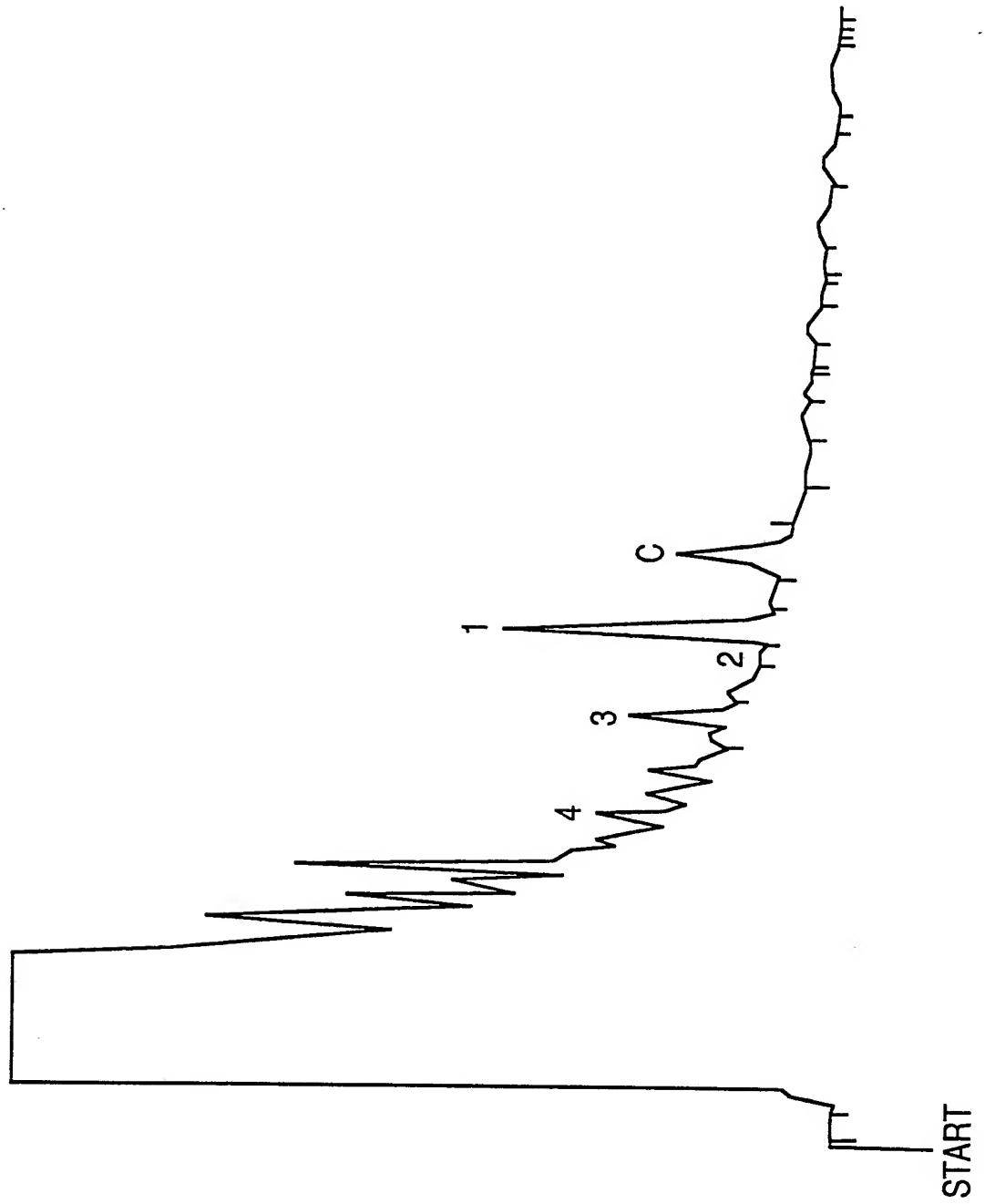


FIG. 3

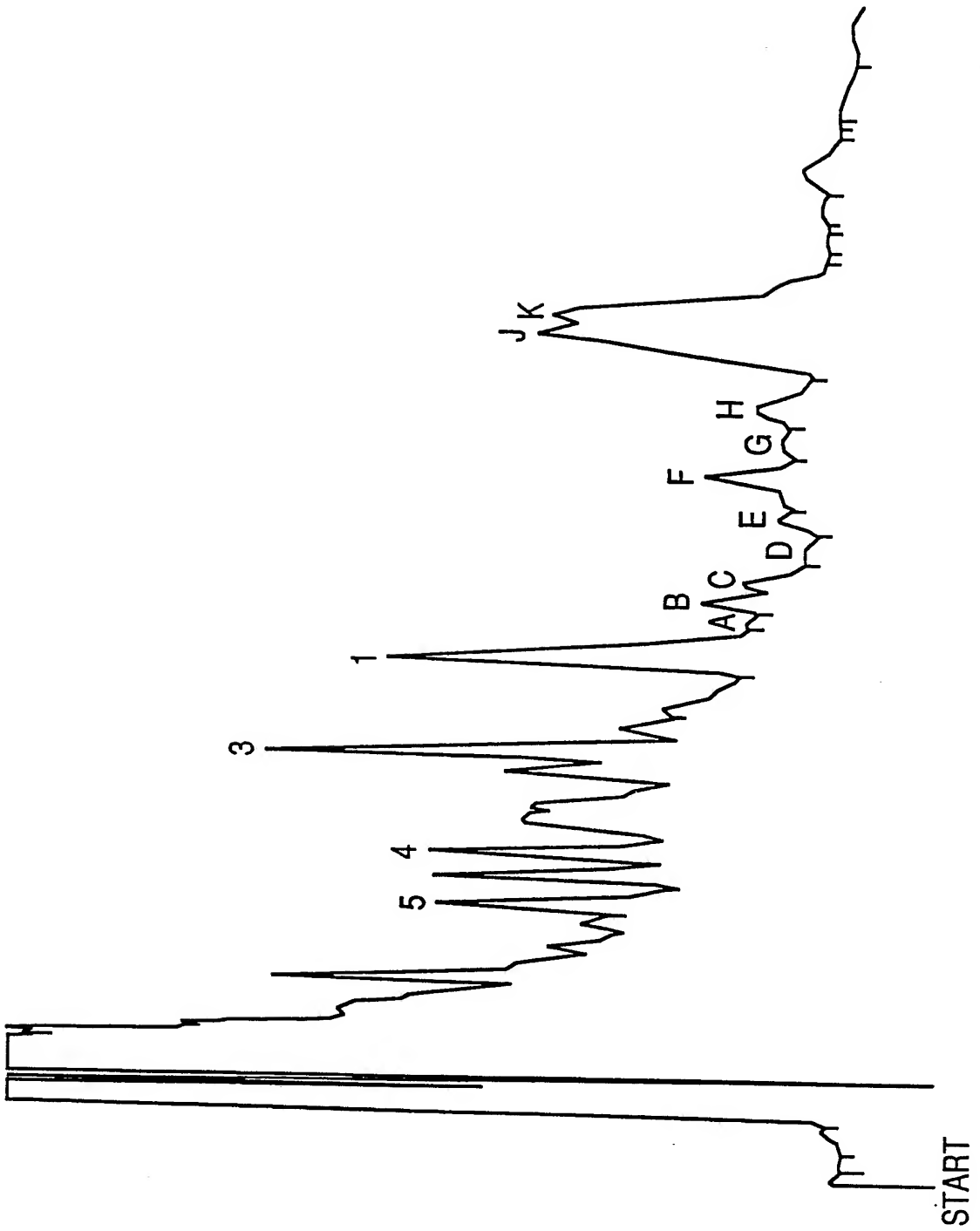


FIG. 4

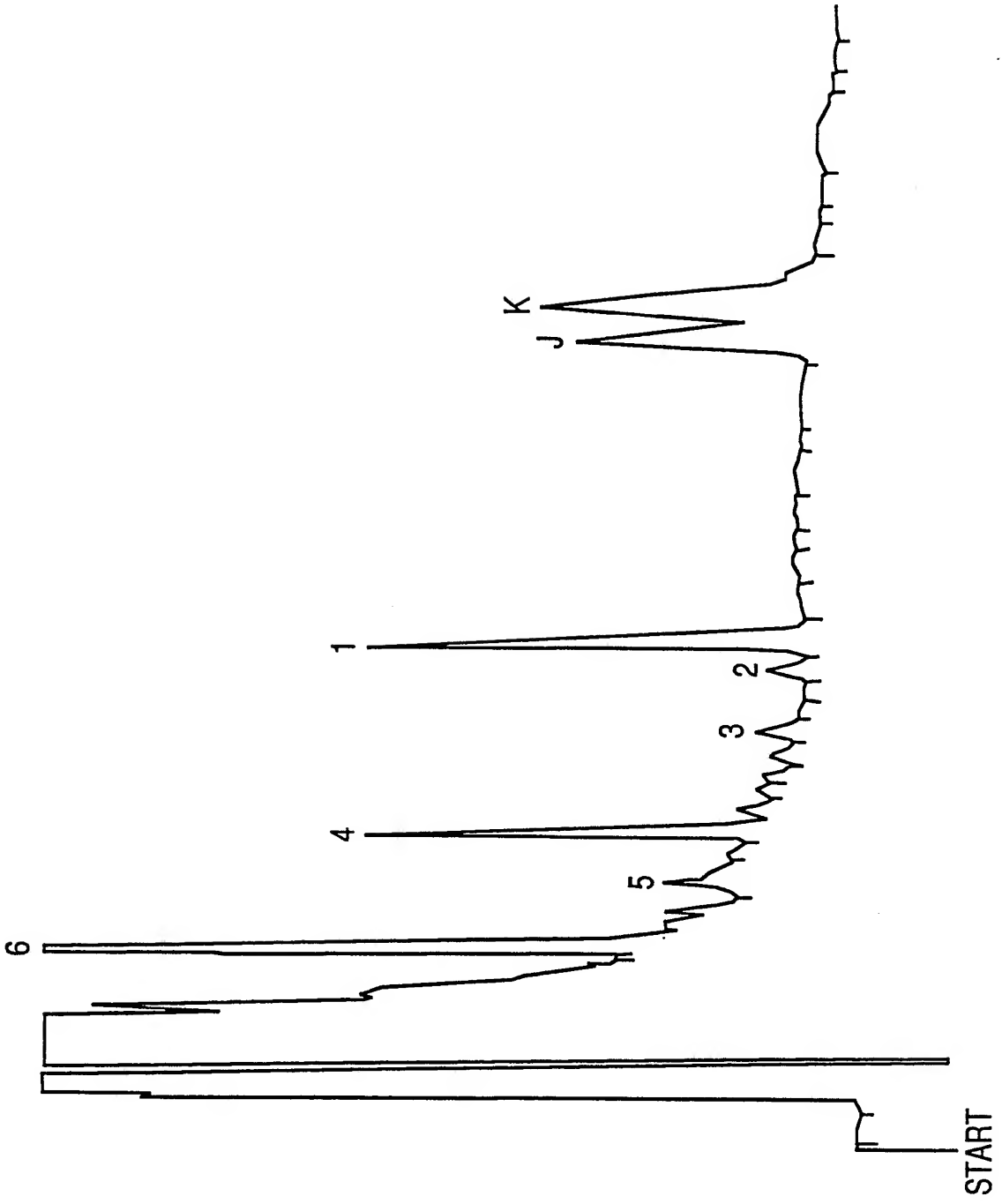
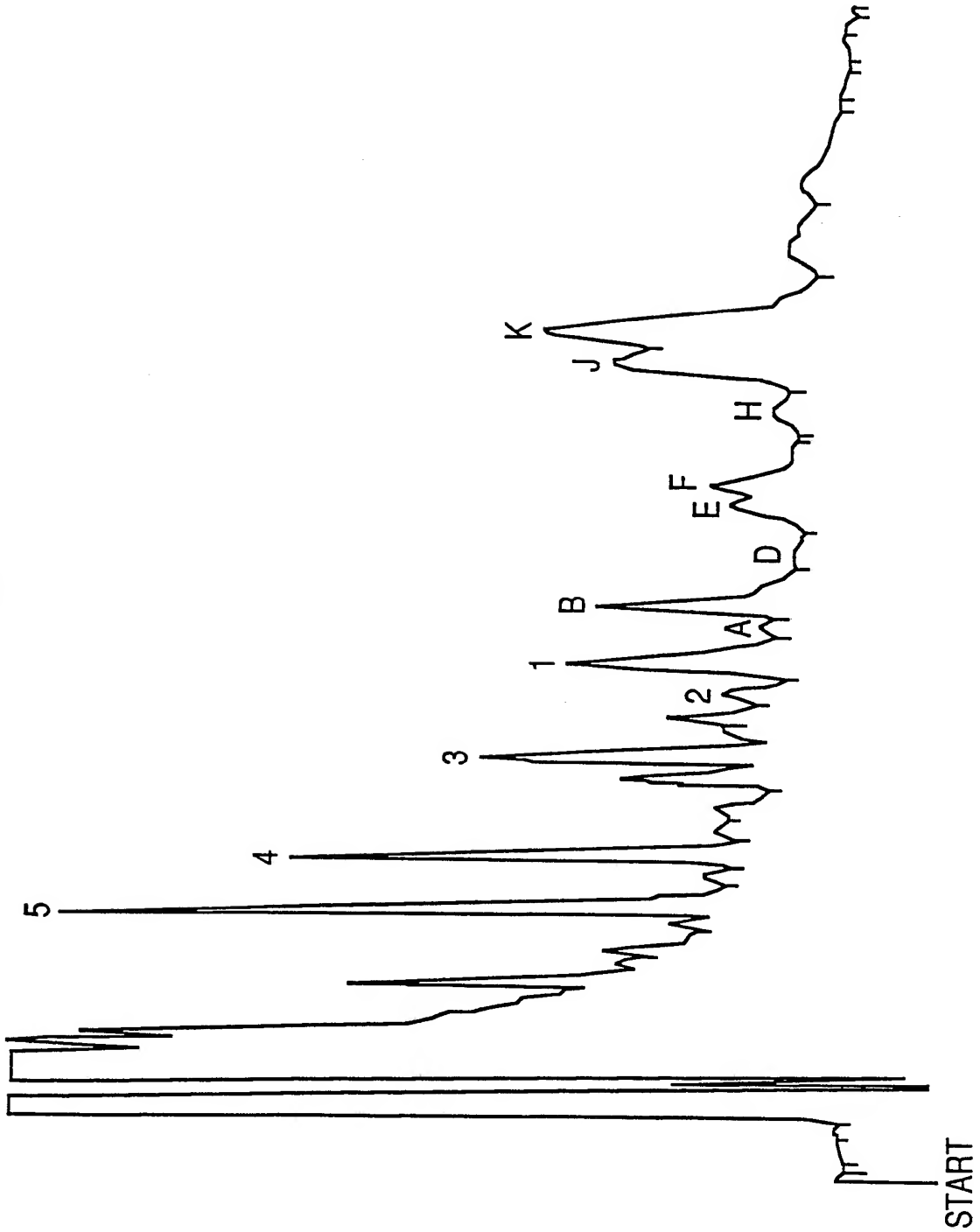


FIG. 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04424

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 17/02, 1/00; C12N 5/02, 5/04; C07D 305/14; A61K 31/335
US CL :435/123, 41, 240.4, 240.46, 240.48; 514/449; 549/510
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/123, 41, 240.4, 240.46, 240.48; 514/449; 549/510

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P Y	US, A 5,019,504 (Christen et al) 28 May 1991, see entire document.	1-4, 8, 11-12, 15-17, 27-29, 31, 32 5-7, 9-10, 13-14, 18-26, 30, 33-38

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
15 June 1993

Date of mailing of the international search report
21 JUL 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer
JEFFREY J SEVIGNY

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04424

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P Y	WO, A, 92/13961 (Saito et al.) 20 August 1992, see entire document.	1-5, 7-8, 11-12, 15-17, 19-24, 27, 29, 31- 32, and 36 6, 9-10, 13-14, 18, 25-26, 28, 30, 33-35, and 37-38
X,P Y	Tertrahedron Letters, Volume 33, No. 36, issued 01 September 1992, L. O. Zamir et al., "Biosynthetic Building Blocks of Taxus canadensis," pages 5235-5236, see entire document.	6, 13, 28 36-38
X,P Y	Planta Medica, Volume 58, issued October 1992, A. G. Fett Neto et al., "Distribution and Amounts of Taxol in Different Shoot Parts of Taxus cuspidata," pages 464-466, see entire document.	28, 36 13, 37-38
Y	Proceedings of the National Academy of Sciences, Volume 77, No. 3, issued March 1980, P. B. Schiff et al., "Taxol Stabilizes Microtubules in Mouse Fibroblast Cells," pages 1561-1565, see entire document.	15-16, 32
X Y	Journal of Liquid Chromotography, Volume 12, No. 11, issued 14 December 1989, K. M. Witherup et al., "High Performance Liquid Chromotographic Separation of Taxol and Related Compounds," pages 2117-2132, see entire document.	28, 36 37-38
Y	US, A, 4,942,184 (Haugwitz et al.) 17 July 1990, see entire document.	28, 36-38
Y	US, A 4,924,012 (Colin et al.) 08 May 1990, see entire document.	28-36-38